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E2F3a Functions as an Oncogene and Induces DNA Damage Response Pathway Mediated Apoptosis

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E2F3A FUNCTIONS AS AN ONCOGENE AND INDUCES DNA DAMAGE RESPONSE PATHWAY MEDIATED APOPTOSIS

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Dedication

TO

My husband Douglas T. Paulson and our future son

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I would like to thank my mentor, Dr. David Johnson, for providing my lab co-workers and me with such wonderful training and learning opportunities. I would not have accomplished what I have without his support and guidance. Most importantly, he served as a good example with his patience, generosity and mild manners. My graduate supervisory committee, Dr. Shawn Bratton, Dr. Edward Mills, Dr. Carla Van Den Berg and Dr. Nomeli Nunez also offered a lot of their time, valuable help and advise to me. I am grateful to all of the past and present members of the Johnson laboratory for their discussions and for being such a friendly crowd. I thank Dr. Mark McArthur for assistance with pathology, Dr. Penny Riggs for her expertise and help in real-time PCR. Ms. Becky Brooks at Science Park Research Division, for her help and being such a cheerful and charming person, Ms. Mickie Sheppard for her tremendous assistance in providing the correct procedures we have to follow to finish our graduate studies. She made these seem so easy and I can't imagine all the headaches the paper work would have caused without her help.

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E2F3A FUNCTIONS AS AN ONCOGENE AND INDUCES DNA DAMAGE RESPONSE MEDIATED APOPTOSIS

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Mutation or inactivation of RB occurs in most human tumors and results in the deregulation of several E2F family transcription factors. Among the E2F family, E2F3 has been implicated as a key regulator of cell proliferation and *E2f3* gene amplification and overexpression is detected in some human tumors. To study the role of E2F3a in tumor development, we established a transgenic mouse model expressing E2F3a in a number of epithelial tissues via a keratin 5 (K5) promoter. Transgenic expression of E2F3a leads to hyperproliferation, hyperplasia and increased levels of p53-independent apoptosis in transgenic epidermis. Consistent with data from human cancers, the *E2f3a* transgene is found to have a weak oncogenic activity on its own and to enhance the response to a skin carcinogenesis protocol. While E2F3a induces apoptosis in the absence of p53, the inactivation of both p53 and p73, but not p73 alone, significantly impairs apoptosis induced by E2F3a. This suggests that both p53 and p73 contribute to E2F3a induced apoptosis but that their function is compensatory. Even though data suggest that E2F3a carries out its unique apoptotic activity in part through another E2F family

member E2F1, unlike E2F1, the ARF tumor suppressor is required for E2F3a-induced apoptosis. While both E2F3a and E2F1 require ATM for apoptosis, E2F3a activates ATM through a distinct mechanism from E2F1. The overexpression of E2F3a results in the accumulation of DNA damage in K5 transgenic keratinocytes and normal human fibroblasts (NHFs). In response to this, the DNA damage checkpoint kinase ATM is activated, and phosphorylation of the downstream targets p53 and the histone variant H2AX are significantly increased. Additional studies show that increased Cdk activity and aberrant DNA replication contributes to DNA damage, ATM activation and apoptosis in response to deregulated E2F3a, which suggest that aberrant replication imposed by deregulated E2F3a plays an important role in the activation of the ATM DNA damage response pathway. Activation of ATM by E2F3a is not affected by loss of ARF or E2F1. Meanwhile, E2F3a-induced ARF upregulation is not affected by E2F1 loss. The above results indicate that E2F3a engages several parallel pathways involving E2F1, ARF and the ATM kinase, and these pathways cooperate to promote apoptosis.

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Chapter I Introduction

1.1 E2F FAMILY

1.1.1 The history of E2F

E2Fs were originally identified by their ability to bind to and activate the adenoviral *E2* gene promoter and were named the E2 binding factor (Kovesdi et al., 1986). *E2* gene activation by E2F requires the function of adenoviral early region 1A (E1A) transforming protein (Bagchi et al., 1990). The retinoblastoma protein (RB) binding domain of E1A is critical for E1A to mediate *E2* gene activation by E2F. This led to the finding that in normal cells, E2F is inhibited by its association with RB. E1A stimulates cell cycle entry by sequestering RB and inducing the release of E2F (Bagchi et al., 1991; Bandara and La Thangue, 1991; Nevins, 1992). E1A and several other viral proteins including the large T antigen of SV40 and the E7 oncoprotein of human papilloma virus all bind to RB and release E2F activity (Dyson et al., 1989; Whyte et al., 1988). E2F controls the transcription of a group of genes that are important for cell cycle progression (Dyson, 1998). The critical role E2F plays in cell cycle control is highlighted by the requirement of E1A to bind RB in order to induce DNA synthesis and to contribute to oncogenic transformation.

E2F also associates with and is regulated by two other members of the RB family, p107 and p130 (Ewen et al., 1991; Li et al., 1993). These proteins belong to the same multigene family and differ in their abilities to interact with different E2F family members. This will be further addressed in the next section.

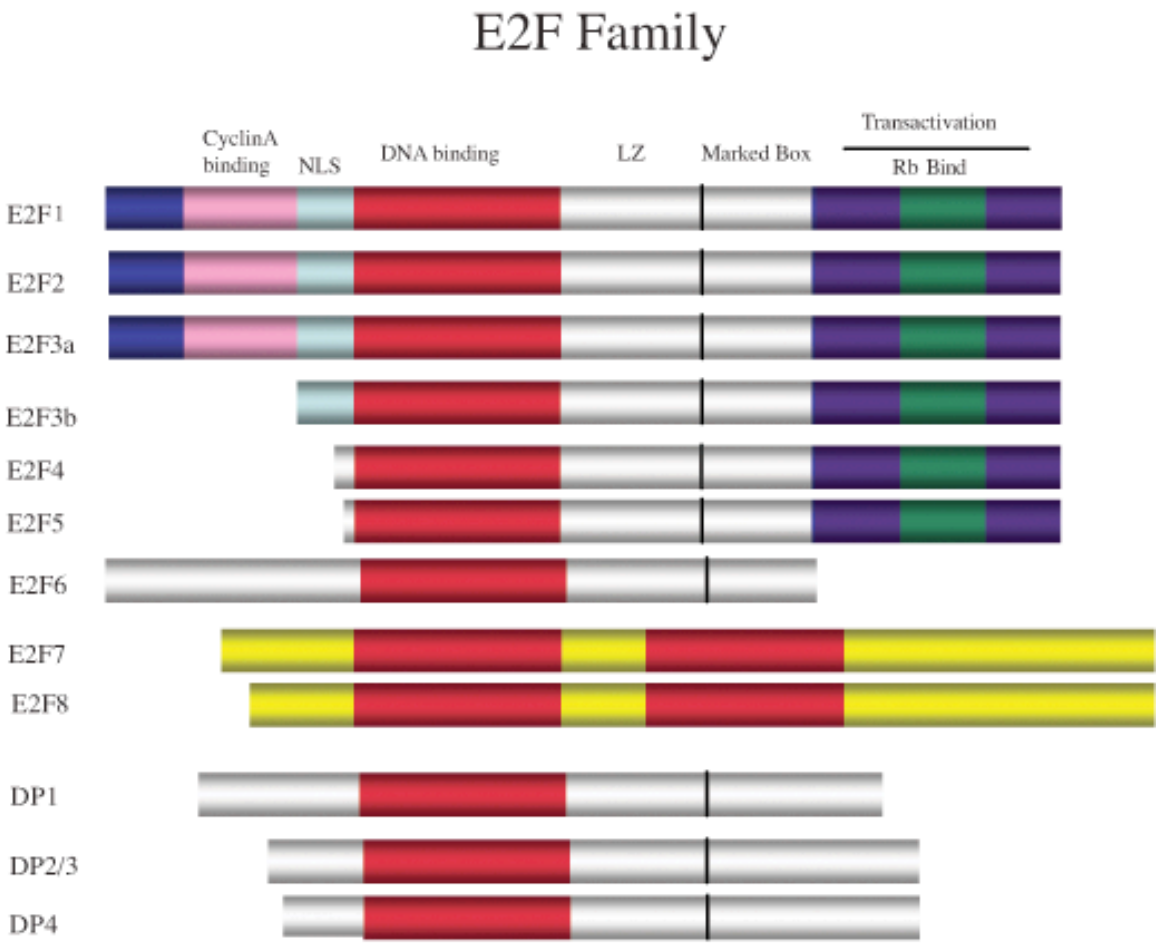
Around the same time when E2F was discovered, a group identified differentiation regulated transcription factor 1 (DRTF1). This factor was down regulated during the differentiation of F9 embryonic carcinoma stem cells (La Thangue and Rigby, 1987). The consensus DNA-binding site of DRTF1 was found to be the same as that of E2F, and DRTF1 also interacted with RB (Bandara and La Thangue, 1991; La Thangue and Rigby, 1987). Now it is clear that DRTF1 and E2F are the same factor. Importantly, during the purification and microsequencing process of DRTF1, a key component of DRTF1/E2F was discovered and called DRTF protein 1 (DP1) (Girling et al., 1993) (Figure 1). DP1 is a heterodimerization partner for members of the E2F family. Although related factors (known as DP2/3 and DP4) can also heterodimerize with E2F, DP1 appears to be the major component of cellular E2F activity (Bandara et al., 1994; Milton et al., 2006; Wu et al., 1995).

1.1.2 Members of the E2F family

The E2F family of transcription factors are critical regulators of cellular proliferation, differentiation, development and apoptosis. A number of E2F family members have been identified, including six E2F (E2F1-6) and three DP (DP1, DP2/3 and DP4) proteins (DeGregori and Johnson, 2006; Dyson, 1998; Helin, 1998; Milton et al., 2006). Recently two new E2F members E2F7 and E2F8 have been reported (de Bruin et al., 2003; Maiti et al., 2005b) (Figure 1). The six distinct genes, E2F1 through E2F6 of the E2F family encode structurally related proteins with highly conserved DNA-binding and dimerization domains. E2F1-6 bind DP proteins to form functional heterodimers, which bind with high affinity to E2F DNA-binding sites in target gene promoters. E2F7 and E2F8 contain two DNA-binding domains but do not dimerize with

Figure 1. The E2F family of transcription factors.

This figure summarizes the structural characteristics of E2Fs. Shown are the domains found in the E2F/DP family members including the cyclin A binding domain; NLS, nuclear localization signal; DNA binding domain; LZ, leucine zipper together with MB, marked box domain mediates dimerization with DP; and transactivation domain which also includes the RB binding domain.



DP proteins and instead may bind DNA as monomers. With the exception of E2F6-8, the carboxy terminus of each E2F protein contains the defined transcriptional activation domain. Embedded within the transactivation domain is a region of homology involved in binding to proteins of the RB tumor suppressor family.

Adding additional complexity to the E2F family, the E2F3 gene encodes two protein products (E2F3a and E2F3b) through the use of alternative promoters at one genomic locus (He et al., 2000; Leone et al., 2000). E2F3a is expressed in proliferating cells with peak expression at the G1/S phase boundary while E2F3b, which lacks amino-terminal sequences found in E2F3a, is expressed constitutively, including in quiescent cells.

The E2Fs have been divided into several subclasses according to their transcriptional regulatory properties on E2F target genes. One subclass includes E2F1, E2F2 and E2F3a, which are referred to as the activator E2Fs because they transcriptionally activate E2F target genes such as thymidine kinase and cyclin E. The activator E2Fs associate predominately with RB and their expression is cell cycle regulated with maximum expression in late G1 phase (Lees et al., 1993). This control is the result of several mechanisms, with one being E2F-dependent transcriptional repression in quiescent cells (Adams et al., 2000; Johnson et al., 1994b; Sears et al., 1997). A second mechanism of regulation is interaction with cyclin A-Cdk2 through the amino terminus cyclin A binding domain and consequent phosphorylation by the cyclin A-Cdk2 complex, which results in decreased DNA binding activity of the E2F-DP dimer (Dynlacht et al., 1997; Kitagawa et al., 1995; Krek et al., 1994). E2F is also subject to ubiquitin mediated protein degradation, though this mechanism is not limited to activator E2Fs, and the association with RB protects E2F from proteolysis (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996). Another subclass of

E2Fs, which includes E2F3b, E2F4 and E2F5, are referred to as repressor E2Fs because their primary function appears to be to repress the transcription of E2F target genes when in association with RB, p107, or p130. This subclass is expressed constitutively but transcriptional repression by these factors primarily occurs in quiescent and early G1 cells. E2F4 and E2F5 nuclear localization appears to depend on association with RB and DP family members (Lindeman et al., 1997; Magae et al., 1996; Muller et al., 1997). E2F4 is predominantly nuclear in quiescent and early G1 cells, translocating to the cytoplasm during progression into S phase. The third subclass, which includes E2F6, 7 and E2F8 also appear to function as transcriptional repressors of E2F target genes but do so independent of the RB family (de Bruin et al., 2003; Maiti et al., 2005a; Morkel et al., 1997).

1.2 E2F AND CELL CYCLE CONTROL

1.2.1 The cell cycle in mammalian cells

The cell division cycle is usually divided into four distinct phases, G1 (gap 1), S (synthesis), G2 (gap2) and M (mitosis) phases (Sherr and Roberts, 1999). Gap phases allow cells time to grow and double their mass of proteins and organelles in order to replicate their DNA and divide. DNA duplication occurs during S phase, which requires 10-12 hours and occupies about half of the cell cycle time in most mammalian cells. After S phase, chromosome segregation and cell division occur in M phase, which requires less than an hour in most mammalian cells.

Growth factors exert their effect during the G1 phase. The restriction (R) point defines a critical time in late G1 after which a cell is committed to DNA replication even if the extracellular signals that stimulate cell growth and division are removed. After the R point, cell cycle progression can only be halted at checkpoints by conditions of cellular stress, such as mitotic-spindle defects or DNA damage.

Cyclin-dependent kinases (Cdks) lie at the heart of the cell cycle control system. Many enzymes and proteins control cyclical changes in Cdk activity. Among them, the most important Cdk regulators are cyclins, which undergo a cycle of synthesis and degradation in each cell cycle. Cyclical changes in cyclin levels result in the cyclic assembly and activation of the cyclin-Cdk complexes; this activation in turn triggers cell-cycle events. Cyclin binding leads to partial activation of Cdk by exposing the active site of Cdk. Cdk-activating kinase (CAK) will then phosphorylate near the Cdk active site resulting in full activation of cyclin-Cdk kinases. The rise and fall of cyclin levels is the primary determinant of Cdk activity during the cell cycle. However, to fine tune Cdk activity at specific stages of the cell cycle, phosphorylation by Wee1 protein kinase inhibits Cdk activity and dephosphorylation by Cdc25 increases Cdk activity. The association and dissociation of Cdk inhibitor proteins (CKIs) can also regulate the activity of cyclin-Cdk complexes.

The sequential activation of Cdk4/6, Cdk2, and Cdc2 (Cdk1) controls the orderly progression of cell cycle through the four phases. In G1 phase after growth factor stimulation, increased cyclin D synthesis occurs and newly synthesized cyclin D assembles with Cdk4/6 into active Cdk4/6-cyclin D complexes. By contrast, the expression of cyclin E is triggered by internal signaling pathways. In the cases of the ordered activation of the remaining cyclin-Cdk complexes, each cyclin-Cdk complex triggers the activation of the next cyclin-Cdk species and also induces its own

destruction. Under conditions of cellular stress, cell cycle progression is disrupted by the activation of checkpoint pathways that ultimately lead to the inhibition of one or more cyclin-Cdk complexes.

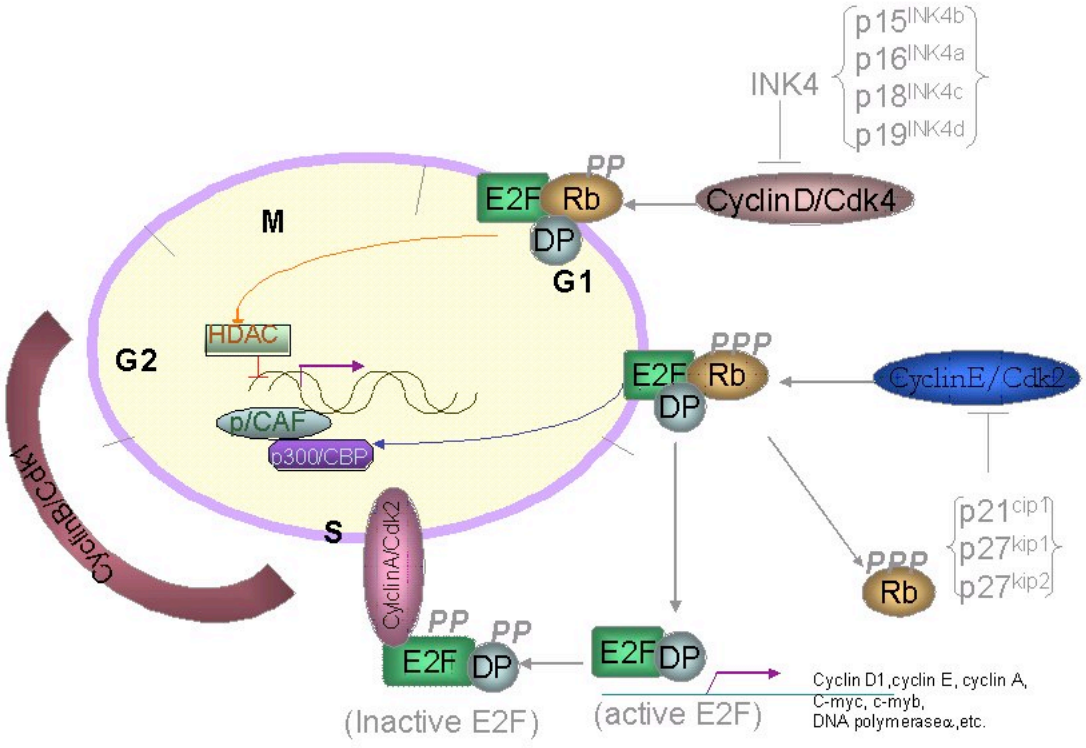
1.2.2 The roles of E2F in cell cycle

The E2F transcription factors regulate a number of the genes that encode products required for cells to progress into S phase from quiescence. These genes encode cell cycle regulators such as cyclin E, cyclin A, cyclin B, Cdc2, Cdc25A and RB; enzymes that are involved in nucleotide biosynthesis such as dihydrofolate reductase (DHFR), thymidylate synthetase and thymidine kinase (TK); and the main components of the DNA-replication machinery, including Cdc6, ORC1 and the minichromosome maintenance (Mcm) proteins (Ishida et al., 2001; Muller et al., 2001; Wang et al., 1999).

The transcriptional activity of E2F1-5 is regulated through association with the RB (retinoblastoma) tumor suppressor protein or the related RB family proteins, p107 and p130 (Dyson, 1998; Helin, 1998). When bound to E2F, RB family members not only block transcriptional activation by E2F-DP heterodimers, but can also actively repress transcription by recruiting histone deacetylases (HDAC) and other chromatin remodeling factors to the promoters of E2F-responsive genes (Brehm et al., 1998; Dyson, 1998). Formation of RB family proteins/E2F complexes is cell cycle regulated. When quiescent cells are stimulated to enter the cell cycle, cyclin D expressed at the G1-phase of the cell cycle activate their associated Cdk4/6, which phosphorylate the RB family proteins (Sherr, 1996; Weinberg, 1995) along with cyclin E-Cdk2 complexes during progression of the cell cycle, resulting in sequential dissociation of HDAC/Rb/E2F complexes. This leads to derepression as well as activation of E2F–regulated genes. Furthermore, free E2Fs have the ability to interact with histone acetyltransgerases (HATs), such as p/CAF

Figure 2. Role of activator E2Fs in cell cycle progression.

Hypophosphorylated RB binds to E2F in early-G1 phase and inhibits its activity. The E2F/DP1/RB complex also actively suppresses the transcription of E2F target genes by recruiting chromatin remodeling enzymes, including histone deacetylases (HDACs). When cells progress to mid-G1 phase, RB is sequentially phosphorylated by cyclin D/Cdk4,6 and cyclin E/Cdk2 and hyperphosphorylated RB releases E2F/DP dimer, which initiates transcription of the genes required for G1/S transition. In this step, E2F interacts with p300/CBP or p/CAF to recruit histone acetylase activity to the target promoter and activate gene transcription.



or p300/CBP which act as co-activators to promote transcription at the target promoters (Martinez-Balbas et al., 2000; Marzio et al., 2000). These events result in an ensuing S phase entry (Figure 2).

1.2.3 Activator E2Fs trigger S phase entry.

E2F1, E2F2, and E2F3a are potent transcriptional activators of E2F-responsive genes (Dynlacht et al., 1997; Helin et al., 1992). It is a well established that the activator E2Fs are important in regulating gene expression at the G1/S phase transition of the mammalian cell cycle (Botz et al., 1996; Dyson, 1998; Leone et al., 1998) and are important for cell cycle progression (Bagchi et al., 1990; Lavia and Jansen-Durr, 1999). When overexpressed, all these activator E2Fs have the ability to induce quiescent cells to enter S phase and positively regulate cell proliferation. In some contexts, E2F4 and 5 also can induce S phase when overexpressed (DeGregori et al., 1997; Johnson et al., 1993; Lukas et al., 1996; Vigo et al., 1999). Ectopic expression of E2F1 can also override various growth arrest signals, including TGF-β, DNA damage and the Cdk inhibitors p16, p21, p27 (DeGregori et al., 1997; Mann and Jones, 1996; Schwarz et al., 1995). Moreover, overexpressed E2F1, E2F2 and E2F3a can induce transformation of an established cell line (Singh et al., 1994; Xu et al., 1995), or transform primary rat embryo cells in cooperation with other oncogenes such as an activated *Ras* oncogene (Johnson et al., 1994a). More importantly, transgenically expressed E2F1 and E2F3a can induce tumor development in mouse models (Paulson et al., 2006; Pierce et al., 1998a; Pierce et al., 1999).

Endogenous activator E2Fs also have been shown to play important roles in the control of cellular proliferation. The combined loss of all activator E2Fs is sufficient to

completely block cellular proliferation (Wu et al., 2001), which indicates that the activator E2Fs have overlapping roles in the induction of cell cycle entry, and subsequently cell proliferation. In agreement with these results, inhibition of endogenous E2F3 by microinjection of anti-E2F3 antibodies causes cell cycle arrest in primary cells (Leone et al., 1998) *E2f3*-deficient mouse embryonic fibroblasts (MEFs) have a reduced rate of proliferation in both primary and transformed cells caused by defective activation of almost all known E2F responsive genes in response to mitogen stimulation (Humbert et al., 2000). Furthermore, in these E2F3 null mouse models, E2F3 loss has a stronger negative effect on cell proliferation compared with the loss of E2F1 and E2F2 (Humbert et al., 2000; Leone et al., 1998). Therefore, E2F3a is considered to be a strong positive regulator for cell proliferation. It was also shown that following mitogenic induction in primary fibroblasts, E2F3a activates most known E2F-responsive genes whose products are rate limiting in DNA replication such as Cdc6 and the Mcm proteins (Hateboer et al., 1998; Kowalik et al., 1998). Thus, E2F3a is a crucial factor that determines the rate of proliferation in primary fibroblasts (Humbert et al., 2000).

1.3 E2F AND APOPTOSIS

1.3.1 Activator E2Fs induce apoptosis

In addition to inducing proliferation, deregulated E2F activity triggers apoptosis in mammalian cells and transgenic mouse systems. Ectopic expression of *Drosophila* E2F (dE2F) in flies also induces apoptosis (Asano et al., 1996; Du et al., 1996). Concerning the function of E2F in apoptosis induction, some consider it a specific function of E2F1, while others disagree. Early findings show that when overexpressed in

REF52 fibroblasts, the apoptotic induction function is unique to E2F1 (DeGregori et al., 1997; Hallstrom and Nevins, 2003). Supporting data show that ectopic expression of E2F1 leads to apoptosis in cultured cells (Kowalik et al., 1995; Shan and Lee, 1994; Wu and Levine, 1994) and transgenic mice (Holmberg et al., 1998; Pierce et al., 1998a; Pierce et al., 1999; Shan and Lee, 1994). Further studies have shown that inactivation of RB in mouse brain epithelium induces aberrant proliferation and apoptosis that are suppressed by E2F1 deficiency (Pan et al., 1998).

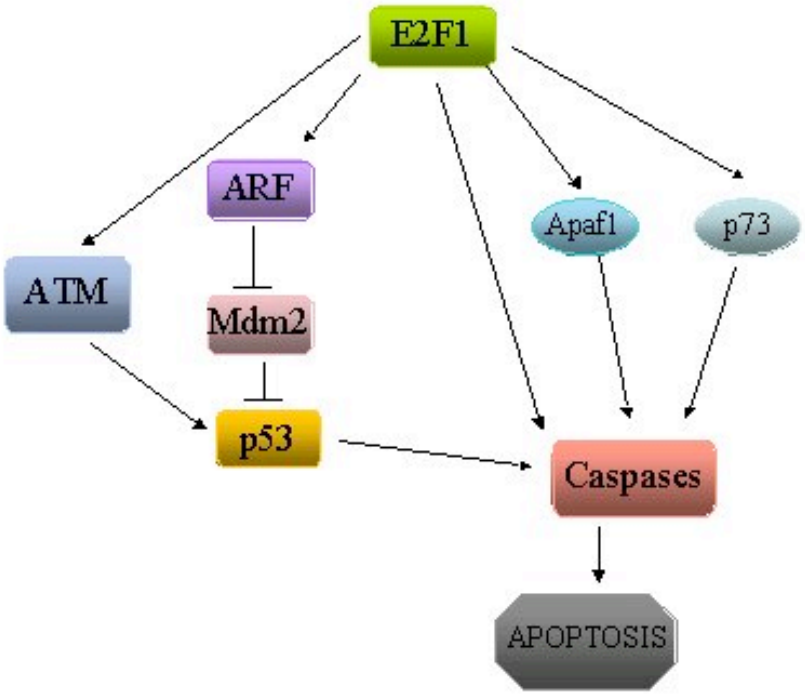
Conversely, other experiments using either overexpression systems or loss of function systems suggest other activator E2Fs trigger cells undergo apoptosis as well. The ectopic expression of estrogen receptor (ER) regulated fusions with E2F1, E2F2 or E2F3 all induce apoptosis in Rat1 fibroblasts (Vigo et al., 1999). A report shows that the E2F1 marked box domain has a unique proapoptotic activity among E2Fs (Hallstrom and Nevins, 2003). On the other hand, our unpublished results show that introduction of a mutation in the marked box domain of E2F3a significantly reduces the level of apoptosis induced by overexpression of E2F3a. Also, E2F1 or E2F3 deficiency, but not E2F2 deficiency, can rescue the inappropriate apoptosis observed in several tissues in mouse embryos with inactivated RB (Saavedra et al., 2002; Tsai et al., 1998; Ziebold et al., 2001). These findings indicate that deregulated E2F3 and possibly E2F2 induce apoptosis and E2F3 also plays a role in apoptosis that occurs in the absence of RB. While ectopic expression of E2F3 was reported to induce apoptosis in some experimental cell systems (Vigo et al., 1999) but not in others (DeGregori et al., 1997; Kowalik et al., 1998), our recent results together with others from different transgenic mice systems show overexpression of E2F3 induces apoptosis *in vivo* (Lazzerini Denchi and Helin, 2005; Paulson et al., 2006; Vigo et al., 1999). These systems provide more reliable results that

E2F3 does induce apoptosis, since the animal system provides a more physiologically relevant environment and largely avoids the variations in different cell culture systems.

1.3.2 Apoptosis pathways by E2F

The mechanisms underlying E2F-induced apoptosis are quite complex, but can be classified as either p53-dependent (Kowalik et al., 1995; Shan and Lee, 1994) or p53-independent pathways (Hsieh et al., 1997; Irwin et al., 2000). In p53-dependent apoptosis, one mechanism underlying the accumulation of p53 induced by deregulated E2F1 expression is the direct transactivation of the *p19Arf* tumor suppressor gene (*p14Arf* in human) by E2F1 (Bates et al., 1998; Palmero et al., 1998). ARF interacts and sequesters Mdm2 E3 ubiquitin ligase to nucleoli (Pomerantz et al., 1998; Weber et al., 1999; Zhang and Xiong, 1999) and blocks nucleo-cytoplasmic shuttling of Mdm2 (Tao and Levine, 1999; Zhang and Xiong, 1999), resulting in the inhibition of Mdm2's function. By inhibiting Mdm2, ARF inhibits the ability of Mdm2 to target p53 for ubiquitination and subsequent degradation (Weber et al., 1999). Thus, E2F1 induced increase in ARF levels leads to dissociation of Mdm2 from p53 and results in p53 stabilization. The level of p53 is normally maintained at low levels because Mdm2 is a transcriptional target of p53 and thus participates in a negative feedback loop. However, several reports demonstrate that deregulated E2F1 induces apoptosis in a p53-dependent but ARF-independent manner (Russell et al., 2002; Tsai et al., 2002). In the absence of ARF, E2F1-induced apoptosis is correlated with p53 phosphorylation by the ATM kinase (Rogoff et al., 2002; Rogoff et al., 2004) (Figure 3). The mechanism of how E2F1

Figure 3. E2F mediated apoptosis pathways.
In the classical model, E2F1 upregulates the transcription of the *Arf* tumor suppressor gene, which interferes with Mdm2 mediated p53 degradation and results in increased p53 level. Activation of p53 leads to the transcription of its downstream targets such as p21 for cell cycle arrest and proapoptotic factors bax, Noxa, Puma and other genes that promote apoptosis. More recent findings provide evidence that ATM is responsible for E2F-induced apoptosis. The deregulation of E2F1 in some way activates ATM and causes it to phosphorylate a series of downstream effectors including Chk2 and p53, leading to p53 stabilization and activation and consequently apoptosis. In p53-independent apoptosis, E2F1 upregulates the activity of the p53 homologue p73, which activates p53 responsive genes to promote apoptosis. Overexpression of E2F1 also upregulates proapoptotic factors such as Bak, Bad and Bid, effector caspases including caspase 3 and 7, and Apaf-1, which activates caspase 9 and eventually activates effector caspase 3 and 6.



activates ATM is under extensive study.

Besides p53-dependent apoptosis introduced above, E2F1 also induces p53-independent apoptosis. The p53 homologue p73 is believed to be directly activated by E2F1, leading to activation of p53 responsive genes and apoptosis (Irwin et al., 2000; Stiewe and Putzer, 2000). Also DNA microarray studies show that ectopic expression of E2Fs up-regulate expression of proapoptotic members of the Bcl-2 family, such as Bok, Bad, Bak and Bid1, and effector caspases, including caspase-3 and caspase-7 (Ma et al., 2002; Muller et al., 2001). Induction of E2F1 activity also results in an increase of mRNA and protein levels of Apaf1, which activates caspase-9 and consequently caspase-3 and caspase-6 (Moroni et al., 2001). ARF can also function as a tumor suppressor independently of p53 (Weber et al., 2000), by inducing apoptosis through Bax (Suzuki et al., 2003), or by inhibiting the processing of rRNA (Sugimoto et al., 2003). As well, ATM has recently been shown to phosphorylate proapoptotic Bcl-2 family member Bid in response to DNA damage, suggesting a possible mechanism for ATM- induced p53-independent apoptosis (Kamer et al., 2005; Zinkel et al., 2005).

While the activities of E2Fs are regulated by posttranslational control through protein-protein interactions, they are also subject to transcriptional control. This transcriptional control is partially regulated by one or more E2F family members. It has been shown that E2F3 induces E2F1 expression by binding and activating its promoter (Araki et al., 2003; Neuman et al., 1994; Wells et al., 2000). The ability of E2Fs to regulate each other provides one explanation for the phenomenon that the E2F transcription factors have their specific roles in cell proliferation, apoptosis, differentiation and development, yet share some redundant functions. Recently, it was shown that increased apoptosis mediated by deregulated E2F3 is dependent on endogenous E2F1 (Denchi et al., 2005). Since E2F family members inter-regulate each

other, and overexpression of E2F3 upregulates the mRNA level of E2F1, it would be interesting to know how depriving endogenous E2F1 will affect E2F3a-induced apoptosis in our transgenic mouse model.

1.4 ATM AND THE DNA DAMAGE RESPONSE

1.4.1 Oncogene induce DNA damage and the DNA damage response

Recent reports show that in several types of human tumors, the ATM DNA damage response pathway is activated. In carcinomas of the lung and breast, there is constitutive activation of Chk2 (DiTullio et al., 2002), an effector kinase that is activated by ATM (Ataxia Teleangiectasia Mutated) in response to DNA double-strand breaks (DSBs). The early precursor lesions of human urinary bladder, breast, lung and colon tumors and experimentally induced skin hyperplasia with overexpression of growth factors all express DNA damage response markers. These include phosphorylated kinases ATM and Chk2, phosphorylated histone H2AX and p53, p53 accumulation and formation of 53BP1 foci (Bartkova et al., 2005; Gorgoulis et al., 2005). Apoptosis is also observed in these precursor lesions. Three more recent studies report that oncogene-induced senescence is also mediated by the DNA damage checkpoint pathway (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). During the complicated process of tumor development, many factors contribute to the formation of cancer. To determine the factors that evoke the DNA damage response observed in the above cancerous lesions, studies have shown that oncogenes such as E2F1 and Myc cause DNA damage in cultured cells (Lindstrom and Wiman, 2003; Vafa et al., 2002). Furthermore, a series of studies have also demonstrated in cell culture experiments that a number of oncogenes such as cyclin E, Cdc25A, E2F1 and Myc, when overexpressed, induce the

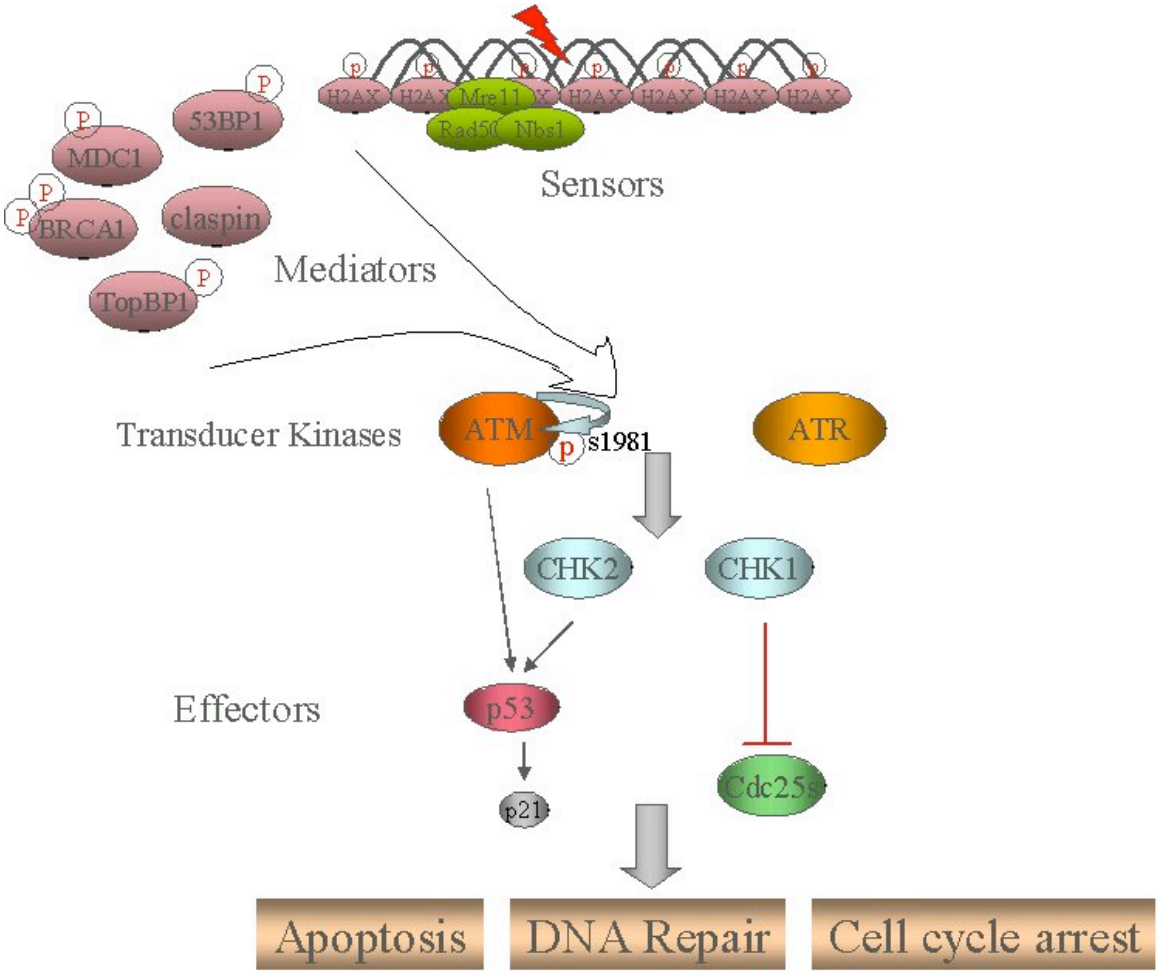
phosphorylation of several DNA damage checkpoint markers. These include serine 15 phosphorylated p53, γH2AX, serine 966 phosphorylated SMC1 and Threonine 68 phosphorylated Chk2 (Bartkova et al., 2005; Bartkova et al., 2006; Gorgoulis et al., 2005; Powers et al., 2004; Rogoff et al., 2004).

It has been proposed that activation of the ATM signaling pathway by oncogene-induced damage mediates tumorigenesis barriers that slow or inhibit the progression of preneoplastic lesions to neoplasia. One such barrier is apoptosis or cell cycle arrest (Bartkova et al., 2005; Gorgoulis et al., 2005), while a second barrier is oncogene-induced senescence (Bartkova et al., 2006; Mallette et al., 2007). Cells with an intact DNA damage response frequently arrest or die in response to DNA damage, thus reducing the likelihood of progression to malignancy. Mutations in apoptosis, DNA-damage responses or in mitotic-checkpoint pathways, can permit the survival or the continued growth of cells with genomic abnormalities, thereby enhancing the chance of malignant transformation. Senescence was initially identified as a response of tissue culture cells to eroded telomeres or activated oncogenes (Lowe et al., 2004). The relevance of senescence to human cancer was unclear until its recent demonstration in preneoplasia (Collado et al., 2005; Michaloglou et al., 2005). Oncogene-induced senescence has been associated with increased expression of the tumor suppressors $p16^{INK4A}$ and ARF (Lowe et al., 2004; Narita et al., 2003; Serrano et al., 1997). Recent studies have shown that activation of the DNA damage checkpoint pathway is critical in mediating oncogene-induced senescence (Bartkova et al., 2006; Mallette et al., 2007). Taken together, data from human cancer tissue and from cell culture studies suggest that deregulated oncogenes have the ability to induce DNA damage relatively early in cancer development and activate DNA damage responses that result in halted cell cycle progression, programmed cell death or senescence.

1.4.2 The DNA damage response signaling pathways

In response to DNA damage, the activation of PI(3)K (phosphatidyl-inositol-3 kinase)-like kinases (PIKKs) such as ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3- related) are one of the first steps characterized to date in the activation of DNA damage checkpoint pathways. ATM primarily responds to DNA breaks while ATR plays a critical role in the response to cellular stress that inhibits replication fork progression. Because many types of DNA damage result both in the direct damage of the DNA and the arrest of DNA replication forks, ATM and ATR participate together in many cellular stress responses. Here we emphasize the role of ATM in response to DNA double-strand breaks (DSBs). ATM is a serine/threonine kinase with a 350 kilodalton molecular weight. ATM is not essential for critical cellular functions such as cell cycle progression or cellular differentiation, because humans, mice or cells that lack ATM are viable (Shiloh and Kastan, 2001). ATM kinase is mostly activated when cells are under stresses that affect DNA or chromatin structure. DNA-double strand breaks are recognized by sensors as the Mre11-Rad50-Nbs1 (MRN) complex, which then recruits ATM to sites of damage. In normal cells, ATM exists as a homodimer in which the kinase domain is physically blocked. The introduction of double-strand breaks (DSB) lead to a conformational change in ATM, which induces intermolecular autophosphorylation on serine 1981. The phosphorylation of ATM on serine 1981 causes dissociation of the homodimer and results in activated monomeric ATM (Bakkenist and Kastan, 2003). Activated ATM phosphorylates histone H2AX on its C-terminus (termed γH2AX) and also phosphorylates other mediator proteins such as 53BP1, MDC1, TopBP1, and BRCA1. The recruitment of mediator proteins to the site of DNA breaks

Figure 4. DNA damage response pathways.
At the site of DNA double-strand breaks (DSB), before the activation of the response network, DSB sensors, including MRE11, RAD50, and NBS1 that form MRN complexes, are rapidly recruited to the site of damage. The sensors convey a damage signal to transducers, for example ATM and ATR, which in turn phosphorylate the C-terminus of histone H2AX at serine 139 and elicit coordinated recruitment and phosphorylation of mediators to the DNA damage site. The mediators enhance and amplify the signal of transducer kinases and cause the phosphorylation of numerous downstream effectors, which contributes to cell cycle arrest, DNA damage repair or apoptosis.



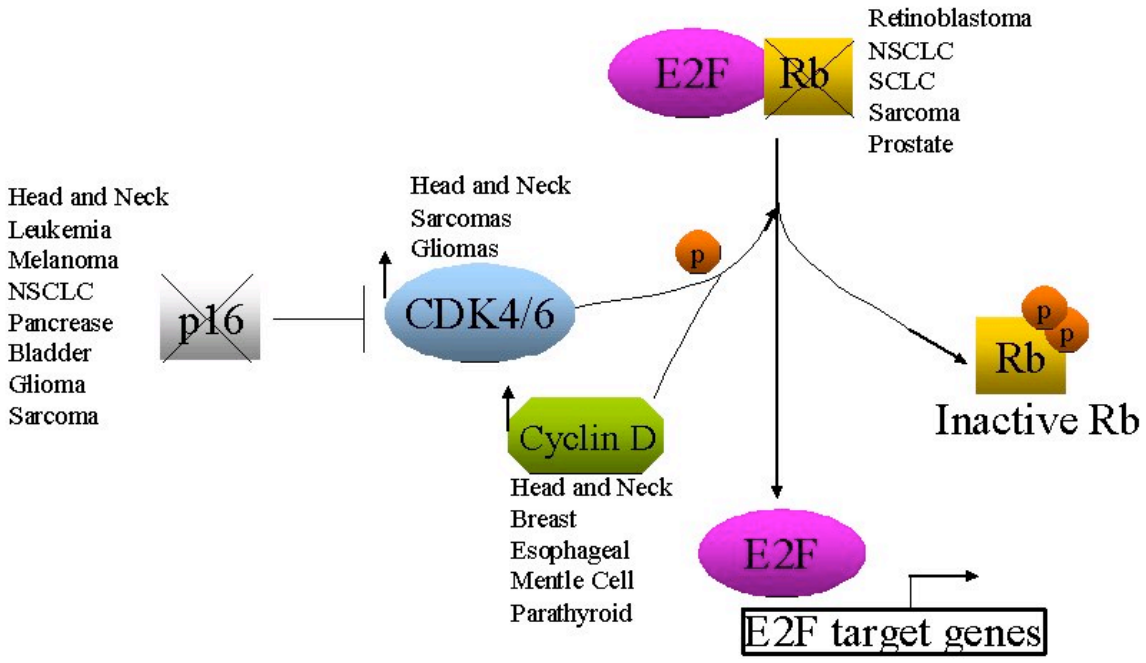
facilitates ATM kinase to phoshorylate downstream target effectors such as Chk2, p53 and SMC1. The above series of processes lead to the biological outcomes of the checkpoint responses including cell cycle arrest, apoptosis, and DNA damage repair (Figure 4).

1.5 THE LINK BETWEEN E2F AND CANCER

1.5.1 Rb/E2F pathway and cancer

While RB interacts with many proteins, the critical targets of RB cell cycle repressive activity are the E2F transcription factors (Morris and Dyson, 2001; Nevins, 2001). Even though E2Fs are rarely mutated in human tumors, there is much evidence to suggest that the activation of E2F transcription factors, via perturbation of the $p16^{INK4a}$-cyclin D/Cdk4,6-RB tumor suppressor pathway, is a key event in the development of most cancers (Figure 5). RB was the first tumor suppressor to be identified and its loss of function is found in the majority of all human tumors (Weinberg, 1992). In untransformed cells, without mitogenic factor stimulation, RB binds to E2F and inhibits E2F responsive genes, and consequently prevents the initiation of DNA replication and cell cycle progression. When there is *Rb* loss of function, E2F will be released to activate E2F responsive genes and induce S phase entry and cell proliferation. The RB/E2F pathway is regulated directly by cyclin D-Cdk4/6 and indirectly by the $p16^{INK4a}$ Cdk inhibitor. The second most common mutation that happens in human cancers is *p16* mutation. The p16 protein inhibits cyclin D-Cdk4 activity, and in the absence of p16, increased Cdk activity leads to RB phosphorylation and E2F accumulation. Thus, loss of p16 activity is functionally equivalent to RB loss of function. Activating mutations in

Figure 5. Activation of E2F by disturbances of the p16^{INK4a}-cyclin D-retinoblastoma tumor suppressor pathway in cancer.
The retinoblastoma tumor suppressor, RB is inactivated in many human tumors. At the same time, almost all RB positive tumors carry either activating mutations in the *cyclin D* or *Cdk4* genes or inactivating mutations in the Cdk4 inhibitor, *p16^{INK4a}*. The inactivation of RB can occur through roughly three mechanisms: loss or mutation of the *Rb* gene, inactivation by the binding of oncoproteins of DNA tumor viruses, or the deregulated phosphorylation of RB mediated by the aberrant activation of cyclin D or Cdk4 or the loss of the Cdk4/6 inhibitor *p16^{INK4a}*.



the *cyclin D* or *Cdk* genes can also occur in cancer and leads to increased activity of cyclin D-Cdk4 activity, increased phosphorylation of RB and release of E2F.

E2Fs not only transactivate cell cycle-related genes but also repress gene expression. Furthermore, E2Fs are important in regulating both cell proliferation and antiproliferative processes such as apoptosis and senescence. How E2F3a's cell proliferative function and apoptosis induction function are balanced and affect the outcome of tumor development is of great interest. In a mouse model with *Rb*-deficient tumors, E2F3 loss results in suppression of pituitary tumors but promotes the development and metastasis of medullary thyroid carcinomas (Ziebold et al., 2003). This suggests that E2F3 acts as either an oncogene or a tumor suppressor in different *Rb* mutant tumors. Several studies have shown increased E2F3a gene amplification and increased expression in human cancers, including transitional cell carcinoma of the bladder, prostate cancers and retinoblastoma (Feber et al., 2004; Foster et al., 2004; Oeggerli et al., 2004). Also, E2F3a is used as a marker for large-cell lung cancer but not for other types of lung cancers (Borczuk et al., 2003). Previous studies in mice have shown that the E2F transcription factors have different effects in tumor development (Pierce et al., 1999; Wang et al., 2000). To determine what roles E2F3a plays in tumorigenesis, we will use a K5 E2F3a transgenic mice model to study if E2F3a induces tumor development with and without carcinogenic pressures.

When RB is hypophosphorylated, it binds to the activator E2Fs (E2F1, 2, 3a), and negatively regulates their function of promoting cell cycle progression from G1 to S phase. Thus, loss of functional RB leads to inappropriate release of the activating E2Fs, and increased expression of E2F-regulated genes. Using an overexpression system to study E2F3a imitates the physiological condition of RB loss of function. One of the approaches to study individual E2F target specificity is to use overexpression systems to

monitor the transcription of E2F responsive genes and correlated phenotypes. E2F transcription factors appear to play contrasting roles in proliferation and apoptosis, tumor suppression and oncogenesis, with each E2F family member having distinct and partially redundant functions. Here we use *in vitro* and *in vivo* overexpression systems to imitate the loss of RB function in physiological systems to explore the specific roles and mechanisms E2F3a plays in cell proliferation, apoptosis and tumorigenesis.

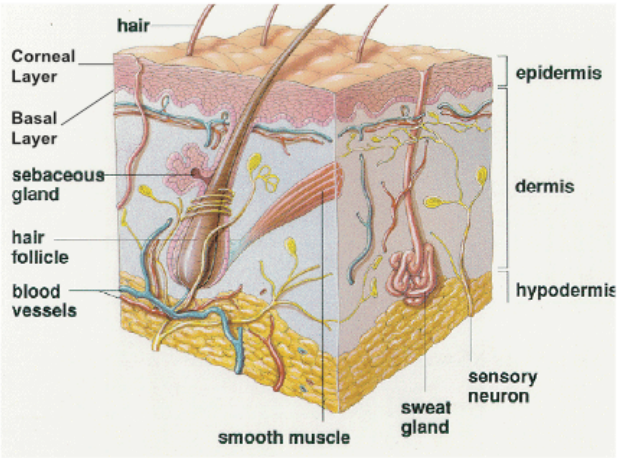
1.6 SKIN CARCINOGENESIS MODEL

1.6.1 Skin keratinocytes undergo constant proliferation and differentiation.

The skin is composed of two major compartments that are separated by a basement membrane. The compartment under the basement membrane is called the dermis, a dense connective tissue layer that plays a supportive role in skin function. The compartment lying above the basement membrane is called the epidermis. The epidermis consists of several layers of keratinocytes: the innermost basal layer to the outermost stratum corneum (Figure 6). The keratinocyte is the predominant cell type in the skin epidermis. The function of keratinocytes includes formation of a mechanical barrier, defense against pathogens and other noxious agents from the outside, and preventing water loss.

One of the important features of epidermis is its self-renewing nature. This depends on constant proliferation of cells in the basal layer to produce new keratinocytes and their differentiation and outward migration. A careful analysis of the growth potential of human skin keratinocytes revealed three different types of cells based on the

Figure 6. The structure of human skin.
The skin is composed of epidermis and dermis. The two sections are separated by a basement membrane. The dermis is a dense connective tissue layer that plays a supportive role in skin function. The epidermis consists of several layers of keratinocytes, from the innermost basal layer to the outermost stratum corneum. The basal layer contains proliferating keratinocytes and the corneal layer contains mostly differentiated, dead cells. The skin also contains appendages such as hair follicles, sebaceous glands and sweat glands. The skin stem cells reside in the bulge region of the hair follicle.



From: www.agen.ufl.edu/~chyn/age2062/lect/lect_19/lect_19.htm

size of the clones they are capable of generating in a single plating: holoclones, meroclones and paraclones (Barrandon and Green, 1987). Holoclones, which have the greatest proliferative potential, contain cells that almost all go on to form proliferative colonies on passaging. It is tempting to speculate that the holocone generating cells *in vitro* might be keratinocyte stem cells *in vivo* which provide the virtually unlimited proliferative capability of skin keratinocytes. Making use of the slow cycling nature of stem cells, researchers have been able to locate keratinocyte stem cells (label-retaining cells) in the mouse skin. The majority of them reside in the bulge region of the hair follicle (just below the sebaceous gland), with a small fraction in the basal layer of interfollicular epidermis (Cotsarelis et al., 1990; Morris and Potten, 1994). It is believed that the stem cells in the bulge region are multipotent and those in the basal layer of interfollicular epidermis are progeny, or unipotent progeny of multipotent bulge cells (Alonso and Fuchs, 2003).

The regulation of keratinocyte proliferation is an extremely complex process. It depends on the availability of growth factors, degree of cell differentiation, and cell attachment to the substrate. Abnormalities of keratinocyte proliferation are of pathogenic significance in many skin disorders. Overexpression of growth factors such as epidermal growth factor (EGF) family members and IGF-1 may be common mechanisms shifting skin keratinocyte into pathological proliferative pathways. Under inflammatory conditions, the interleukins seem to play an important role in activating the pathological pathway of keratinocyte proliferation. *In vitro,* some interleukins are equally potent as EGF in stimulating keratinocyte proliferation (Gniadecki, 1997). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment or UV irradiation also causes keratinocyte proliferation. Curiously, the bulge cells require repeated treatment with TPA to induce proliferation, where as basal layer stem cells are more easily induced to

divide (Morris and Potten, 1999), suggesting that the basal layer stem cells are important in the initial skin response to outside stimuli.

When the population of basal cells expands as a result of constant cellular proliferation, some cells detach from the basement membrane and begin to move outward toward the skin surface. These cells are eventually sloughed from the skin surface after undergoing terminal differentiation, a process that shares some similarities with apoptosis (Gandarillas, 2000). Although this process remains poorly understood at the molecular levels, much knowledge has been obtained from *in vitro* models of keratinocyte differentiation. It is well known that incubation of cultured keratinocytes with high concentrations of calcium (above 0.1 mM) leads to keratinocyte differentiation. It is believed that calcium activates the G-protein-coupled calcium-sensing receptor and leads to the activation of phospholipase C, which in turn produces two important second messengers diacylglycerol and inositol triphosphate (IP3) (Bikle et al., 2001). IP3 stimulates the release of calcium from intracellular stores, resulting in increased intracellular free calcium concentrations (Ca_i). The increase in diacylglycerol and Ca_i activate several isoforms of protein kinase C. Protein kinase C in turn activates the AP-1 family of transcription factors, which regulate the expression of the genes that are involved in keratinocyte differentiation (Bikle et al., 2001).

1.6.2 Skin carcinogenesis is a multistage process.

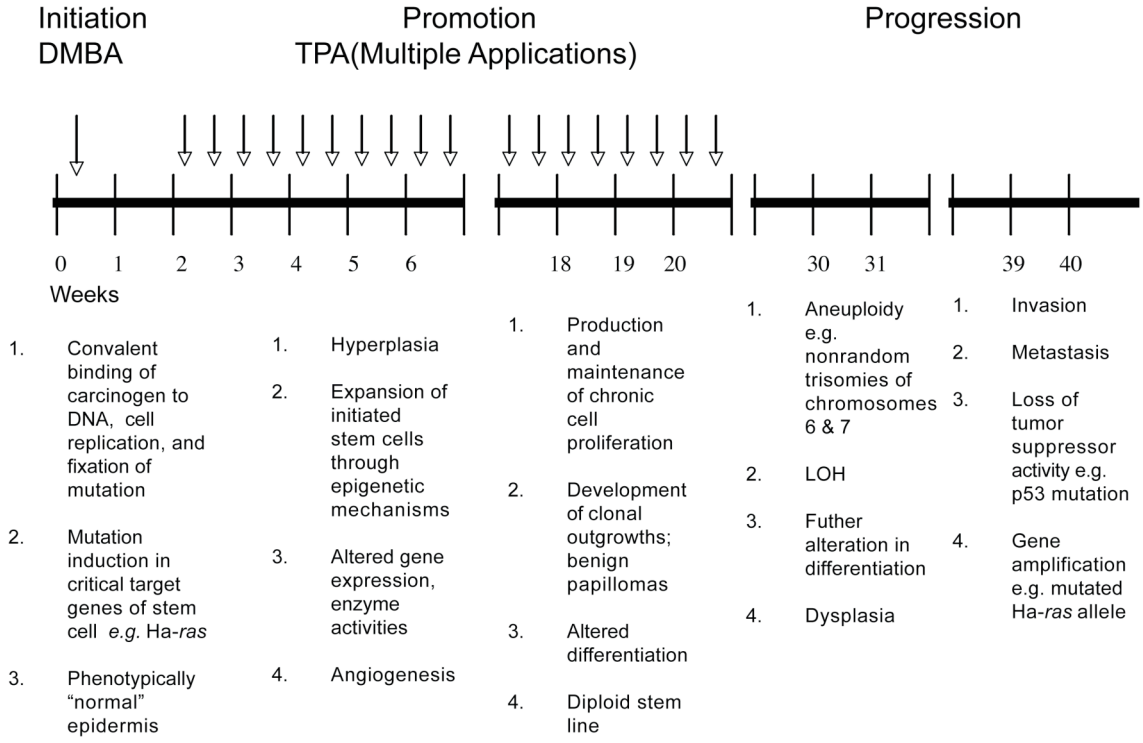
The mouse skin model of carcinogenesis has been instrumental in developing many concepts currently applied to human neoplasias, including the idea that cancer develops through a multistep process that involves many genetic alterations (Bikle et al., 2001; Conti, 1994; DiGiovanni, 1992; Yuspa, 1998). It is often referred to as the "two-

stage" skin carcinogenesis model, but it actually consists of three mechanistic stages termed initiation, promotion, and progression (Figure 7) (DiGiovanni, 1992).

Initiation is generally carried out by application of a single dose of a carcinogen to the dorsal mouse skin. In most carcinogenesis protocols, the initiation step utilizes a chemical agent 7,12-dimethylbenz[a]anthracene (DMBA) or physical agents such ultraviolet (UV) and ionizing radiation (IR). These agents usually cause permanent genetic changes such as mutations, deletions or translocations in a critical gene. Not all cells in the animal exposed to initiating agents would be initiated even if all cells incurred DNA damage and mutation. An initiated cell has to have proliferative potential and cannot have the normal potential to terminally differentiate. The most frequent genetic change in the DMBA-initiated mouse skin is a mutation at codon 61 of the *H-ras* oncogene, which cause it to be constitutively active (Balmain and Brown, 1988; Quintanilla et al., 1986). In most cases, the initiated cells gain a growth advantage compared to normal cells.

Initiation alone, however, is not sufficient for the development of visible tumors during the lifespan of the animal. The selective clonal expansion of the initiated cells in the promotion stage occurs as a result of exposure of the initiated skin to repetitive treatments with an irritating, non-mutagenic agent such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Tumor promotion involves prolonged and repeated application of promoter agents and producing biochemical and cellular responses typical of gene deregulation. For example, the activation of protein kindase C, EGF receptor, ErbB2 and c-Src occurs in response to TPA treatment in mouse epidermis and ODC, c-Fos and c-Myc expression are increased (Conti, 1994; DiGiovanni, 1994; Yuspa, 1998). The endpoint of the promotion stage is the formation of squamous papillomas, which are exophytic, noninvasive lesions. Papillomas in early development (in the first ten weeks)

Figure 7. Multistage model of skin carcinogenesis.
Cancer development involves a multistep process. This model provide an example and consists of three stages. Initiation involves a single dose of a known carcinogen such as DMBA applied to the dorsal skin, which causes a specific genetic change in a critical gene. In the promotion stage, the selective clonal expansion of initiated cells is achieved by repeated exposure of the initiated skin to a non-genotoxic agent such as TPA. Progression is characterized by the conversion of a subset of benign tumors into malignant cancers involving genomic instability, gene amplification and further mutagenesis.



From: DiGiovanni, J (1992) Multistage carcinogenesis in mouse skin. *Pharmacology and Therapeutics* 54, 63-128.

will regress without continued treatment with the promoter but in later stages, the development of papillomas become independent of the promoting agent. Upregulation of cyclin D1 and E2F factors, along with other cell cycle regulators correlates with promoter independence in these later stages (Rodriguez-Puebla et al., 1998; Zhang et al., 1997).

Progression is characterized by the conversion of benign papillomas into malignant squamous cell carcinomas with phenotypes of rapid growth, invasiveness and angiogenesis. Genomic instability, *ras* gene amplification and *p53* mutations are common during progression (Yuspa, 1998). The conversion process occurs spontaneously without the need of continuous tumor promoter application.

In this dissertation, I will first characterize the role of deregulated E2F3a in tumor development. To determine this, a K5 E2F3a transgenic mouse model will be developed and subject to different tumor studies. A spontaneous tumor study is a useful assay to determine the oncogenic property of E2F3a. Further study in two-stage skin carcinogenesis assay will help characterize the effect of deregulated E2F3a on tumor development in response to chemical carcinogen. I will also determine if E2F3a has an apoptotic activity *in vivo,* and if so, I will further determine the pathways by which E2F3a induces apoptosis.

Chapter II Materials and Methods

2.1 Generation of K5 E2F3a transgenic mouse lines.

The K5 E2F3a transgene was constructed by inserting a 1.6 kb cDNA fragment encoding full-length human *E2f3a* into the SnaBI site of pBS-KS-BK5 plasmid, which contains the bovine K5 promoter, the rabbit β-globin intron 2, and the simian virus 40 polyadenylation signal (Ramirez et al., 1994). Founder transgenic mice were made by microinjecting the purified transgene into the pronuclei of zygotes and then implanting the zygotes into pseudo-pregnant female mice. Lines were established and maintained by backcrossing the founders to the FVB strain.

K5 E2F3a transgenic mice (line E2F3.5) in the FVB background were bred to mice containing an inactivated *Atm* allele to generate K5 E2F3a mice heterozygous for *Atm*. F_1 transgenic mice heterozygous for *Atm* were then crossed to *Atm* heterozygous mice to generate K5 E2F3a transgenic and non-transgenic siblings that were either wild type, heterozygous, or nullizygous for *Atm*. The same procedures were used to generate K5 E2F3a transgenic and non-transgenic mice that were either wild type, heterozygous, or nullizygous for *Arf* or *E2f1*. After 6 weeks of age, sibling mice were sacrificed and skin sections were collected for analysis.

2.2 BrdU incorporation.

Mice at 6-8 weeks of age were injected intraperitoneally with 170ul of 20mM bromodeoxyuridine (BrdU) and allowed to incorporate it for 20 minutes before being sacrificed. Lower dorsal skin samples were fixed in formalin, paraffin embedded, and

sectioned. Immunohistochemistry staining was performed with an antibody to BrdU (Becton Dickinson) at 1:500 dilutions. Interfollicular basal layer keratinocytes were examined microscopically to determine the percentage of BrdU incorporation by calculating the ratio of stained and unstained cells. At least 1000 cells were counted.

2.3 ACTIVATED CASPASE 3 IMMUNOHISTOCHEMISTRY.

Skin sections were fixed in formalin, paraffin embedded and sectioned. After deparaffinization and hydration, sections were incubated with antibody specific for the activated form of caspase-3 (R&D Systems, Inc.) at 1:2000 dilutions for 30 minutes. Then the slides were washed and incubated with secondary anti-rabbit antibody and enzyme conjugated using the Histostatin-plus kit (Zymed) for 10 minutes each. Slides were then stained with the kit's diaminobenzidine mixture, rinsed and counterstained with hemotoxylin for 12 to 15 seconds. Apoptosis level were determined by observing the stained slides microscopically and calculated as the average number of activated caspase-3 positive cells per 10 mm of interfollicular epidermis.

2.4 TWO-STAGE MOUSE SKIN CARCINOGENESIS STUDY.

The dorsal skin of 6 to 8 week-old lines K5 E2F3.2 and E2F3.5 transgenic mice and wild type sibling controls were shaved 1 to 2 days before initiation. Mice were initiated with 10nM DMBA in 200 ul of acetone by applying topically to the shaved dorsal skin. Mice were promoted twice weekly with 2.0ug TPA in 200ul acetone applied topically at the same area of DMBA initiation. Mice were scored for papilloma weekly and the ones with skin lesions were included in tabulations until their conditions require

their withdrawal from the study. Tumor samples will be fixed in formalin, paraffin embedded and sectioned for H&E staining. The slides were diagnosed microscopically of their malignancy conversions. Data were analyzed by one-way ANOVA and Pearson Chi-square using SPSS software (SPSS Inc. Chicago, IL).

2.5 REAL-TIME QUANTITIVE PCR.

Total RNA from wild type and E2F3 transgenic mice epidermis tissue was purified with the TriReagent protocol (MRC, Cincinnati, OH). The quality of RNA was checked by on-chip gel electrophoresis (Agilent 2100 Bioanalyzer, Palo Alto, CA). The cDNA synthesis was carried out by reverse transcription with 1μg RNA, 100 units MMLV reverse transcriptase (Ambion, Austin, TX), 40μmol of dNTPs (Ambion, Austin, TX) at 50°C for 95 min in a total volume of 20 μl using 50μM random decamer primers (Ambion, Austin TX). The real time quantitive PCR reactions were performed in 25μl volumes containing 5-10 ng of cDNA, 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 1.25 μl of primers and probes labeled with FAM (Applied Biosystems, Foster City, CA). PCR conditions were 95°C for 10 min to activate the AmpliTaq Gold DNA polymerase, followed by 40 cycles of 95°C for 15s and 60°C for 1min. The test parameter, Ct value, generated by the ABI PRISM 7700 Sequence Detection System software, were analyzed using the arithmetic formula $2^{-\Delta\Delta Ct}$. Mouse GAPDH was used as an internal control gene for normalization. The relative quantitation was calculated by dividing the amount of each target gene by the amount of GAPDH in each sample.

2.6 CELLS AND VIRUSES.

2.6.1 Mouse embryo fibroblasts (MEF) isolation and culture

13-14 day pregnant mice were euthanized and their abdomens soaked with ethanol. The string of embryos (in uterus) was removed from the mother and placed in a 10cm culture dish with 10-20ml PBS.

The following was done one embryo at a time. Each embryo was removed from the uterus. The placenta and embryonic sac were removed. The embryo was placed into 1-2ml clean PBS in a 10cm culture dish separate from the uterus and other embryos. The head was removed and put into a 1.5 ml tube for DNA extraction and genotyping. The blood containing internal organs were removed. The remaining embryo was placed onto the interior side of a 50 ml tube. The embryo was minced finely with scissors. Finally, 10 cold PBS was added to the minced embryo and the tube was placed on ice until all embryos were harvested.

The embryos were then centrifuged (500xG for 5 min) and the PBS was removed by aspiration. Each embryo was resuspended in 7.5 trypsin-EDTA and incubated at 37°C in a water bath for 20 minutes with occasional shaking. Trypsin was then deactivated by adding 7.5ml Dulbecco's modified Eagle's media (DMEM) with 10% fetal bovine serum (FBS) and 1.5xPenicillin/Streptomycin. The embryos were centrifuged again and the supernatant removed by careful pouring. Pellets were resuspended in 12.5ml DMEM, 10%FBS, and then poured through a 70μM filter onto a 10cm culture dish, which was incubated at 37°C, 5%CO_2. Plates were split 1:3 when confluent. This first passage was then frozen for future use or further passaged for experimentation.

All MEF cultures were maintained in DMEM with 10% FBS at 37°C in 5%CO_2. All experiments using MEFs used cells between passage 3 and 6.

2.6.2 Primary human fibroblasts

Primary human dermal fibroblasts from age-, sex-, and ethnicity-matched individuals GM08399 (wild type NHFs) and GM02502 (AT cells from an AT patient) (Coriell Cell Repositories, Camden, NJ) were maintained in MEM with 2mM glutamine, nonessential amino acids, and 15% fetal bovine serum.

2.6.3 Adenoviral infections

Recombinant adenovirus expressing human E2F3a and control virus containing only CMV promoter were made using the AdEasy adenovirus system (Quantum Biotechnologies, Montreal, Canada). For infections, cells were plated at 5000 cells/cm^2 per 10cm plate and cultured overnight. Cultures were then incubated in starvation media containing 0.5% FBS for 24 hours prior to infection. On the day of infection, the cells were washed, counted and cultured in 3 ml of serum-free medium containing AdE2F3a ad AdCMV at the multiplicity of infection (MOI) of 100. After one hour of infection, 7 ml of media with 0.5% FBS was added, and the cells were incubated for an additional 24 hours for the comet assay and 48 hours for apoptosis assay and immunoblotting.

2.7 WESTERN BLOT ANALYSIS.

Epidermal protein was collected by scrapping dorsal skin and resuspending the scrapped off tissue in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM

phenylmethylsulfonyl fluoride, 50 mM NaF, 50 mM Na_3VO_4, 2ug/ml aprotinin, 2ug/ml leupeptin, 1ug/ml pestatin, 13 mg/ml ß-glycerophosphate, and 12 μg/ml sodium vanadate]. Collected samples were frozen in liquid nitrogen immediately and stored in -80°C. Thawed crude samples were centrifuged to be rid of lipid component and unresolved tissue. Centrifuge multiple times if necessary to obtain pure protein samples.

Protein from primary human fibroblasts and embryonic mouse fibroblasts harvested at time indicated post-infection or as described for IR and chemical treatments by scraping cells into either RIPA buffer with protease/phosphatase inhibitors or into 3X SDS loading buffer (as per the Cell Signaling Technology protocol). Protein obtained in RIPA buffer was then quantified using the BCA Protein Assay Kit (Pierce Endogen; no. 23225). Protein obtained in 3X SDS loading buffer was sonicated prior to further steps. Protein samples were then boiled SDS loading buffer and electrophoresis through SDS-polyacrylamide gels, the resolved proteins are transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences) by electroblotting. Specific proteins were detected by standard immunoblotting procedures using the following primary antibodies: E2F1 (KH95, sc-251, Santa Cruz Biotechnology), E2F3 (c-18, sc-878) (Santa Cruz Biotechnology), phospho-Ser15 p53 (9248; Cell Signaling Technology), phopho-Ser1981 ATM (200-301-400; Rockland), γH2AX (05-636; Upstate Biotechnology), p19ARF (200-501-893, Rockland), ß-actin (H-2350; Santa Cruz Biotechnology), ß-tubulin (H-235; Santa Cruz Biotechnology).

2.8 CASPASE-3 ACTIVITY ASSAY.

For caspase 3 activity measurement, cells were washed twice in phosphate-buffered saline and the whole-cell lysate was made in the lysis buffer (50 mM HEPES,

1% Triton X-100, 0.1% 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM DTT, and 0.1 mM EDTA) then, 50-100 μg of whole-cell lysate was added to a reaction mixture containing 50 μM fluorogenic peptide substrates acetyl-DEVD-AFC (Ac-DEVD-AFC) (Biomol, Plymouth Meeting, PA). 50 mM HEPES (pH 7.4), 10% glycerol, 0.1% CHAPS, 100 mM NaC1, 1 mM EDTA, and 10 mM DTT, in a total volume of 100 μl and incubated at 37°C for 1 h. Production of AFC was monitored in a spectrofluorimeter with an excitation wavelength of 400 nm and an emission wavelength of 505 nm. The results were presented as activation increase fold versus that of the control.

2.9 γH2AX IMMUNOFLUORESCENCE.

Formalin fixed, paraffin embedded mouse skin sections were deparaffinized, boiled in10mM sodium citrate for 10 min, and blocked in 50% goat serum for 30 minutes. Sections were then incubated overnight at 4°C with antibody against histone H2AX pS139 (γH2AX) (Upstate Biotechnology) at a 1:300 dilution factor. Samples were washed with phosphate-buffered saline solution for 3 times and incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody from Molecular Probes. The sections were imaged by Olympus BX60 fluorescent microscope.

2.10 IMMUNOHISTOCHEMICAL STAINING FOR P53 PHOSPHO-SERINE 15.

Skin sections were fixed in formalin, paraffin embedded, and sectioned. After deparaffinization and hydration, sections were blocked with 3% H_2O_2 in water for 10 minutes and then washed. Slides were boiled in 10mM citrate buffer pH 6.0 for 15

minutes and after 20 minutes cool down, were blocked in 10% goat serum in phosphate-buffered saline for 30 minutes. Skin sections were incubated with primary p53-phospho-serine15 antibody with a dilution factor of 1:100 (Cell Signaling Technology) for one hour at room temperature. Slides were then washed, incubated with Envision labeled polymer, secondary anti-rabbit antibody conjugated with horseradish peroxidase (Dako) for 30 minutes at room temperature. After buffer wash five times, incubate with Diaminobenzidine (DAB) for five minutes and then wash, counter stain, dehydrate, clear and coverslip slides. Numbers of p53-phospho-serine15 positive cells per 10 mm skin were determined microscopically.

2.11 SINGLE-CELL GEL ELECTROPHORESIS (COMET) ASSAY.

Primary keratinocytes were isolated from newborn mouse epidermis. Skin samples from 2-day-old newborn mice were incubated in trypsin at 4°C overnight, and then the epidermal layer was separated from dermis. Epidermis was then minced with scissors in super media (Gibco, Carlsbad, CA) containing 10% FBS and stirred. Primary keratinocytes were plated at 10^7 / 10cm dish in super media for 2h, then KBM-2 media (Cambrex Bio Science, Walkersville, MD) for 2-3 days. Primary keratinocytes at a concentration of 2 X 10^5 cells per ml were then embedded in low-melting-point agarose on a glass slide by using the Comet Assay kit and the manufacturer's protocol (Reign, Gaithersburg, MD). Briefly, the embedded cells were incubated overnight at 4°C in lysis solution (2.5M NaCl, 100 mM EDTA pH 10, 10 mM Tris, 1% sodium lauryl sarcosinate, 0.01% Triton X-100) followed by incubation in alkaline solution (300 mM NaOH, 1mM EDTA) for 8 minutes to denature the DNA. The samples were then washed twice in 1 X TBE buffer, stained with SYBR Green, and nuclei were visualized by

fluorescence microscopy. Olive tail moments of at least 50 nuclei per slide were scored using COMETSCORE software (TriTek). Student's *t* test was used to derive *P* value.

Chapter III Deregulated E2F3a induces proliferation, tumorigenesis and p53-independent apoptosis *in vivo*

3.1 RATIONALE AND SIGNIFICANCE

Among the E2F transcription factors, E2F1, E2F2, and E2F3a have been referred to as activator E2Fs. They are important in regulating gene expression at the G1/S phase transition of the mammalian cell cycle and are important for cell cycle progression (Bagchi et al., 1990; Lavia and Jansen-Durr, 1999). Several studies done in cell culture systems have shown that overexpression of E2F3 can induce quiescent cells to enter S phase, and subsequently cell proliferation (DeGregori et al., 1997; Johnson et al., 1993; Lukas et al., 1996; Vigo et al., 1999). More evidence of the importance of E2F3 in activating S phase genes and in cell proliferation come from endogenous E2F3 function studies. Mouse embryonic fibroblasts that lack E2F3 have a defect in the mitogen induced activation of almost all known E2F-responsive genes and this results in impaired proliferation of both primary and transformed cells (Humbert et al., 2000). Furthermore, in the E2F3 null mouse models, E2F3 loss has a stronger negative effect on cell proliferation compared with the loss of E2F1 and E2F2 (Humbert et al., 2000; Leone et al., 1998). Therefore, E2F3a is considered to be a strong positive regulator for cell proliferation. It was also shown that following mitogenic induction in primary fibroblasts, E2F3a activates most known E2F-resposive genes whose products are rate limiting in DNA replication such as Cdc6 and the Mcm proteins (Hateboer et al., 1998; Kowalik et al., 1998). Thus, E2F3a is a crucial factor that determines the rate of proliferation in primary fibroblasts (Humbert et al., 2000).

It has been shown that one or more of the activating E2Fs can trigger cells to undergo apoptosis through p53-dependent (Kowalik et al., 1995; Shan and Lee, 1994) or p53-independent mechanisms (Hsieh et al., 1997; Irwin et al., 2000). On the other hand, it was shown that when overexpressed in REF52 fibroblasts, the induction of apoptosis is a specific function of E2F1 (DeGregori et al., 1997; Hallstrom and Nevins, 2003). In other experiments, the ectopic expression of E2F1, E2F2 or E2F3 all induce apoptosis in Rat1 fibroblasts (Vigo et al., 1999). Also E2F3 deficiency severely reduces the inappropriate apoptosis observed in several tissues in mouse embryos with inactivated *Rb* (Saavedra et al., 2002; Ziebold et al., 2001). These findings indicate that deregulated E2F3 induces apoptosis and E2F3 also plays a role in apoptosis that occurs in the absence of *Rb*. While ectopic expression of E2F3 was reported to induce apoptosis in some experimental systems (Vigo et al., 1999) but not in others (DeGregori et al., 1997; Kowalik et al., 1998), it still remains to be answered if ectopic expression of E2F3a induces apoptosis in an *in vivo* system.

Based on the *in vitro* overexpression and loss of function studies, we hypothesized that K5 E2F3a mice would exhibit increased epidermal proliferation. Based on the previous opposite arguments about E2F3a-induced apoptosis, we are interested to determine if E2F3a induces apoptosis in a transgenic mouse model, which provides a more physiological environment for addressing the question of whether E2F3a induces apoptosis. More important, we would like to find out the effect of deregulated E2F3a in tumor development. To determine this, we utilized a K5 E2F3a transgenic mouse model developed in our laboratory. By performing a spontaneous tumor study and two-stage carcinogenesis assay, we seek to determine if E2F3a functions as an oncogene.

3.2 RESULTS

3.2.1 Generation of K5 E2F3a transgenic mice

The *K5 E2f3a* construct was made by inserting 1.6 kb of full length human *E2f3a* cDNA into the SnaBI site of pBS-KS-K5 plasmid, which contains the bovine K5 promoter (Ramirez et al., 1994), the rabbit β-globin intron 2, and the simian virus 40 polyadenylation signal to ensure proper post- translational processing of the gene (Pierce et al., 1998a). The construct is shown in figure 8A (Figure 8A). The bovine K5 promoter fragment directs expression to the basal cell layer of the epidermis, the hair follicles, and other stratified squamous epithelia in transgenic mice (Ramirez et al., 1994). The full-length cDNA was taken from the E2F3a expression vector $pcDNA_3$- E2F3a. Founder transgenic mice were made by microinjecting the purified transgene into the pronuclei of zygotes and then by implanting the zygotes into pseudo-pregnant female mice. Lines were established and maintained by backcrossing the founders to the FVB strain. Two lines of K5 E2F3a transgenic mice that overexpress the E2F3a transgene in squamous epithelium were established from six original founders. Western blot analysis using antiserum that recognizes both mouse and human E2F3 confirmed that these transgenic mouse lines have increased expression of E2F3 protein in epidermal tissue (Figure 8B). Two dominant bands can be observed in western blot analysis, with the upper band corresponding to full-length E2F3a. The lower band may represent an E2F3b-like product that has been shown to be produced from an internal, alternative translation initiation site in the E2F3a mRNA (He et al., 2000). Overexpression of E2F3 was also detected by immunohistochemical staining of transgenic tissue. In the K5 E2F3a transgenic skin, E2F3 protein is found to be localized in the nuclei of keratinocytes in the

Figure 8. Development of K5 E2F3a transgenic mouse lines.

(A) Schematic representation of the *K5 E2F3a* transgene with the bovine K5 promoter, the rabbit β-globin intron 2, full length human *E2F3a* cDNA, and the simian virus 40 polyadenylation signal. (B) Western blot analysis of the epidermal protein lysates from non-transgenic mice (wt) and K5 E2F3a transgenic lines E2F3.2 and E2F3.5 using antisera specific to E2F3 and β-actin. (C) Skin samples from young adult wild type and K5 E2F3a (line3.2) mice were immunohistochemically stained using antisera specific for E2F3. The K5 E2F3a transgenic skin is much thicker compared to wild type skin. The basal layer of K5 E2F3a skin is positive for E2F3 staining while wild type skin lacks staining.

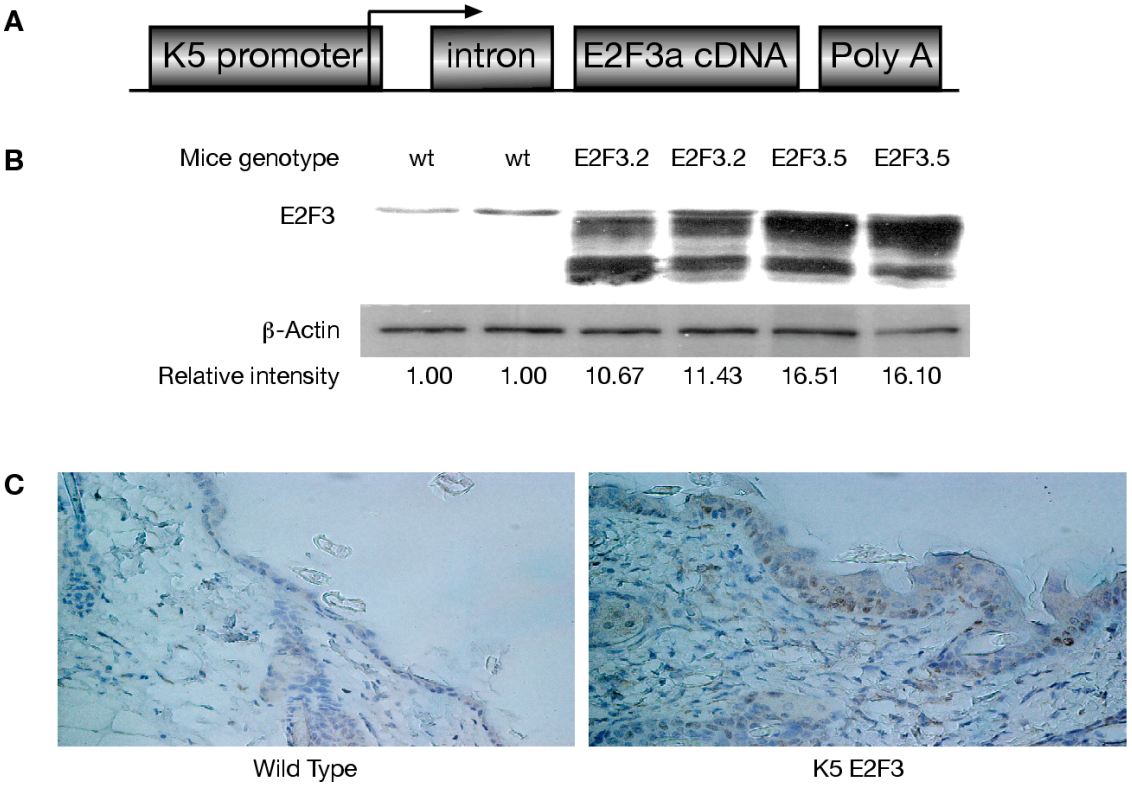
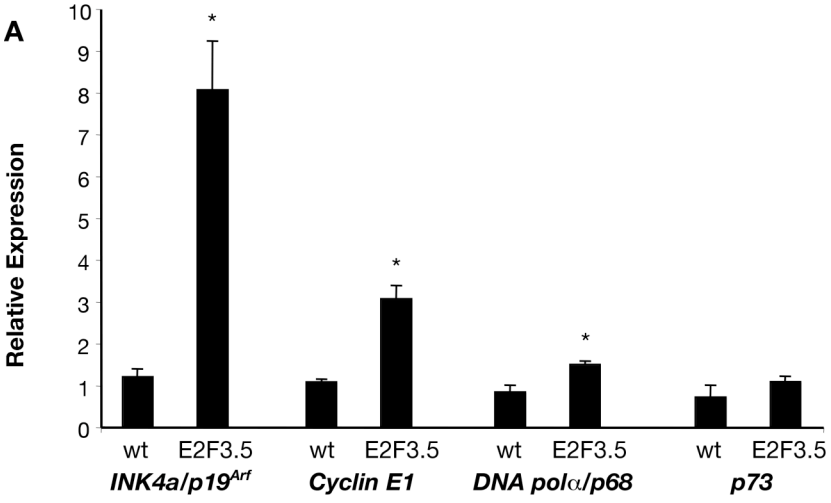
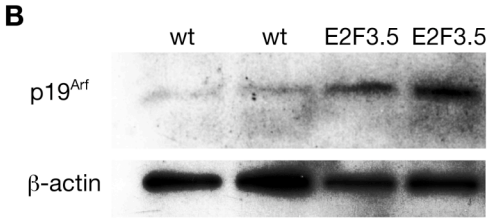


Figure 9. Expression of E2F target genes in K5 E2F3a transgenic mice.

(A) Real-time quantitative PCR was used to measure the expression of *p19^{Arf}, cyclin E, DNA polymerase α/p68, and p73* in RNA samples from the epidermis of K5 E2F3a transgenic (line3.5) and wild type sibling mice. RNA samples from two mice from each genotype were analyzed twice each and normalized to *GAPDH* expression. Bars indicate standard error; * indicates statistical significance where $p < 0.05$ compared to wild type. (B) Western blot analysis of protein extract isolated from epidermis of K5 E2F3a transgenic mice (line3.5) and wild type sibling mice using antisera specific for p19^{ARF} or β-actin.





basal layer of the epidermis and in the outer root sheath of hair follicles (Figure 8C right panel). Our immunohistochemical staining procedure did not detect endogenous E2F3 in nontransgenic tissues from the wild type siblings (Figure 8C left panel).

To determine if our transgene is functional, we tested if E2F3a overexpression resulted in the transcriptional activation of E2F target genes. Real-time PCR was performed on RNA samples isolated from the epidermis of K5 E2F3a transgenic mice and their wild type siblings. The expression of *cyclin E* and *p19Arf* was increased an average of three- and eight-fold respectively in K5 E2F3a transgenic mice compared to wild type controls (Figure 9A). *DNA polymerase α/p68* was also moderately upregulated in K5 E2F3a transgenic epidermis tissue. In contrast, the expression of *p73*, another E2F target gene, was not significantly different between wild type and transgenic mice. This suggests that p73 is a transcriptional target for specific E2F family members such as E2F1 but is not significantly regulated at the transcriptional level by E2F3a. To confirm the increased expression of ARF at the protein level, Western blot analysis was performed using antiserum specific to the exon1β region of the ARF protein (Figure 9B).

3.2.2 Overexpression of E2F3a increases both proliferation and apoptosis

To examine the effect of deregulated E2F3a on cell and tissue homeostasis, skin samples from K5 E2F3a transgenic mice and their wild type siblings were analyzed and compared. The epidermis of both K5 E2F3a transgenic lines was found to be hyperplastic (Figure 10C upper panels) and hyperproliferative (Figure 10C lower panels). The average thickness of the epidermis from line 3.2 and line 3.5 transgenic mice is almost 3 times that of the wild type sibling controls (Figure 10A). The proliferation index of the epidermis was measured by injecting K5 E2F3a transgenic mice and wild type siblings

Figure 10. Hyperplasia and hyperproliferation in K5 E2F3a transgenic epidermis. (A) Epidermal thickness was measured using skin samples from young adult nontransgenic mice (wt) and K5 E2F3a transgenic mice from both lines 3.2 and 3.5. Fifty random measurements were taken on skin samples from five individual mice of each genotype and the average for each genotype is presented. B. Skin samples from the same mice as above were immunohistochemically stained for BrdU incorporation. The percentage of interfollicular basal layer keratinocytes staining for BrdU was calculated for each samples and the average for each genotype is presented. Bars indicate standard deviation; *p<0.05 compared to wild type. C. Representative micrographs of skin samples used in above measurements. Upper panels are H&E staining showing the increased thickness in the two K5 E2F3a transgenic mice lines 3.2 and 3.5. Lower panels are immunohistochemical staining for BrdU showing the increased BrdU incorporation in the basal layer keratinocytes of K5 E2F3a transgenic mice.

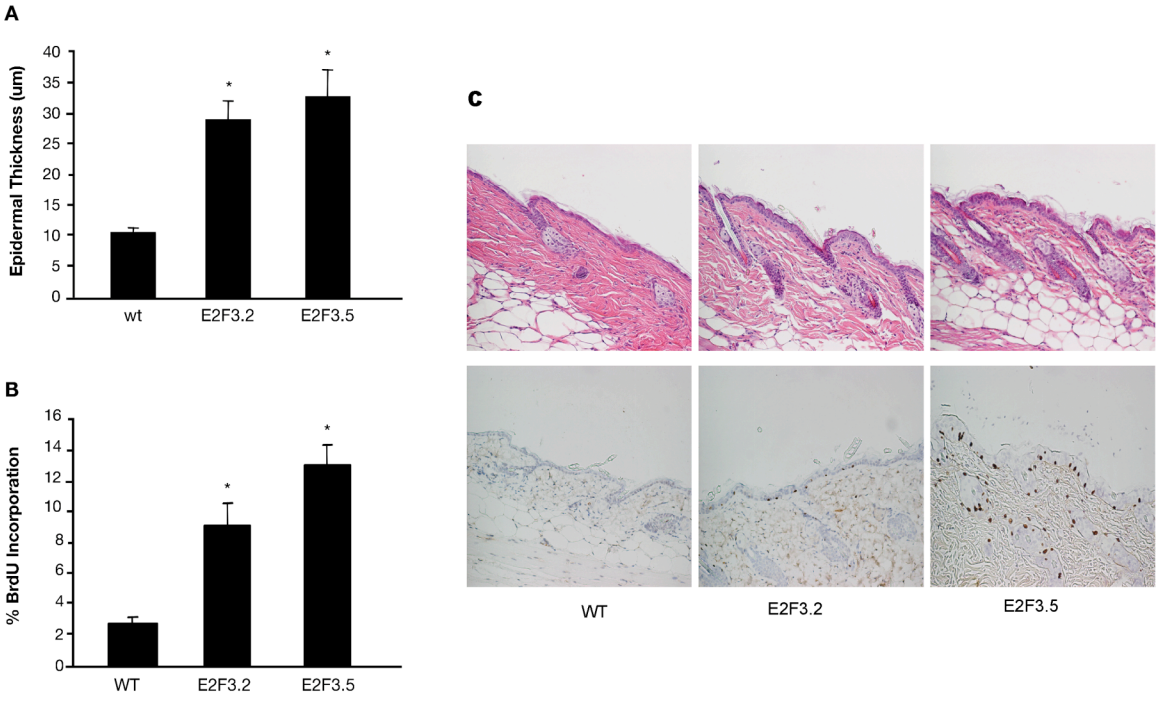
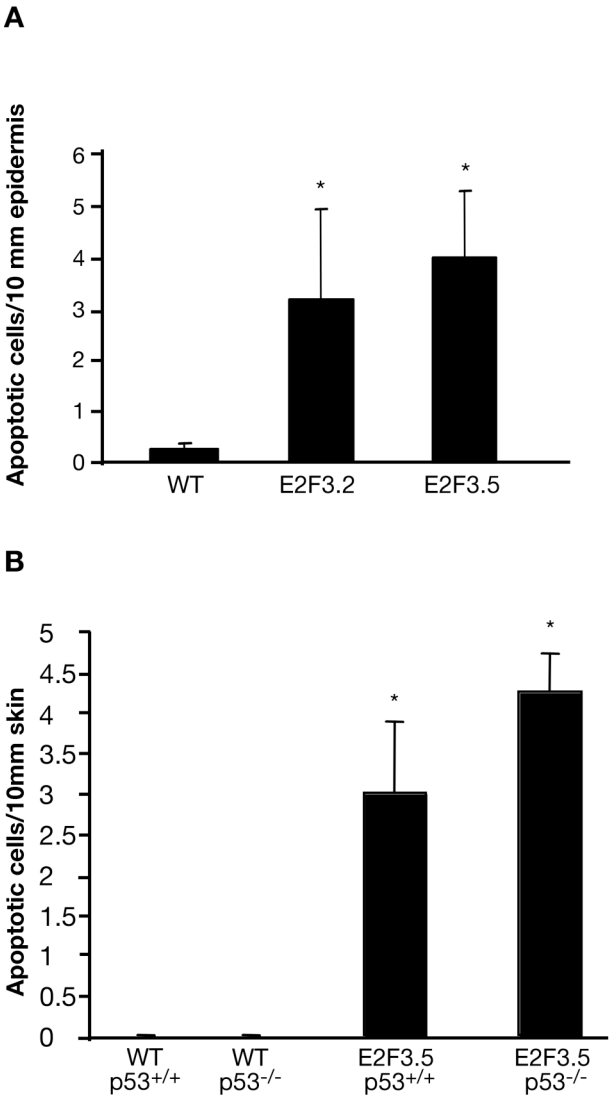


Figure 11. E2F3a-induced p53-independent apoptosis in transgenic epidermis.
(A) Skin samples from non-transgenic (WT) and K5 E2F3a transgenic mice from each line (E2F3.2 and E2F3.5) were immunohistochemically stained for the activated form of caspase 3. The number of caspase 3 positive keratinocytes per 10 mm of skin was determined for five individual mice of each genotype and the average for each genotype is presented. (B) K5 E2F3a transgenic mice (line 3.5) were crossed into *p53* null background and skin samples taken from non-transgenic (WT) and K5 E2F3a transgenic mice with ($p53^{+/+}$) and without ($p53^{-/-}$) functional p53. The average number of caspase 3 positive cells per 10 mm of skin was determined for each genotype as above. Bars indicate standard error; *p<0.05 compared to wild type.



with BrdU prior to sacrifice. Using BrdU incorporation as a marker of S-phase, the number of interfollicular basal keratinocytes in S phase was then determined in skin samples. Approximately three percent of basal layer keratinocytes were in S-phase in nontransgenic mice while the proliferation index of line 3.2 and 3.5 transgenic mice was nine and 13 percent, respectively (Figure 10B and C).

To determine the effect of deregulated E2F3a expression on apoptosis in vivo, epidermal cells expressing activated caspase-3 were detected in skin sections by imunohistochemistry. The number of caspase-3 positive cells in both K5 E2F3a transgenic lines was significantly increased over the level observed in non-transgenic controls (Figure 11A). The TUNEL assay was also performed on skin samples from K5 E2F3a transgenic mice and agreed with the caspase-3 immunostaining results. This feature of E2F3a is similar to E2F1, which when deregulated, also induces elevated levels of apoptosis in mouse epidermis. E2F1-induced apoptosis is dependent on p53 (Pierce et al., 1998b). Even though both E2F3a and E2F1 induced apoptosis, when K5 E2F3a transgenic mice were crossed into a *p53* null background, the absence of p53 had no significant effect on the level of apoptosis observed in K5 E2F3a transgenic epidermis (Figure 11B). This suggests that E2F3a might induce apoptosis using different mechanisms than E2F1.

3.2.3 K5 E2F3a transgenic mice have increased tumor development in both two stage and spontaneous tumor assay

To determine the oncogenic property of E2F3a, a group of K5 E2F3a transgenic mice from both lines was maintained with wild type siblings for two years and monitored for spontaneous tumor development. The tumor incidence of K5 E2F3a transgenic mice

Figure 12. Spontaneous tumor development in K5 E2F3a transgenic mice.

A group of K5 E2F3a transgenic mice from both lines (E2F3.2 and E2F3.5) were maintained for two years along with their non-transgenic siblings (wt). Total tumor incidence (A and B) and incidence of K5-positive epithelial tumors (C and D) are graphed over time for both line 3.2 (A and C) and line 3.5 (B and D).

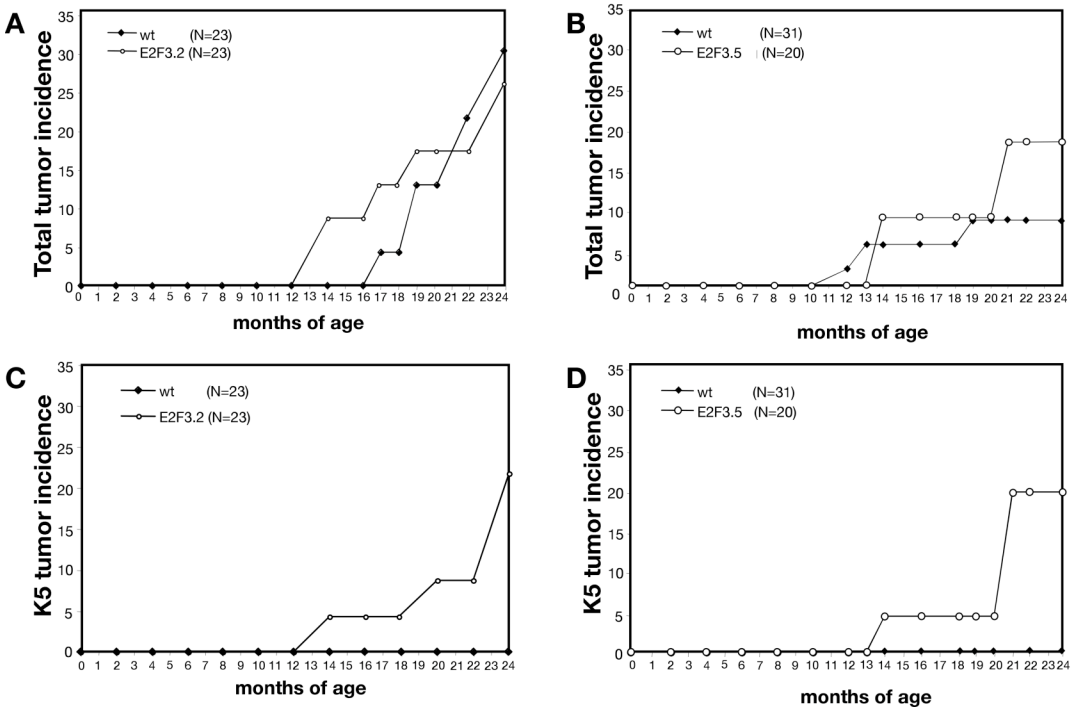


Figure 13. Histological appearance of tumors from K5 E2F3a transgenic mice.

Sections from a mammary carcinoma (A and B) and a squamous cell carcinoma (C and D) were stained with hematoxylin and eosin (A and C) or immunohistochemically stained with antisera to E2F3 (B and D).

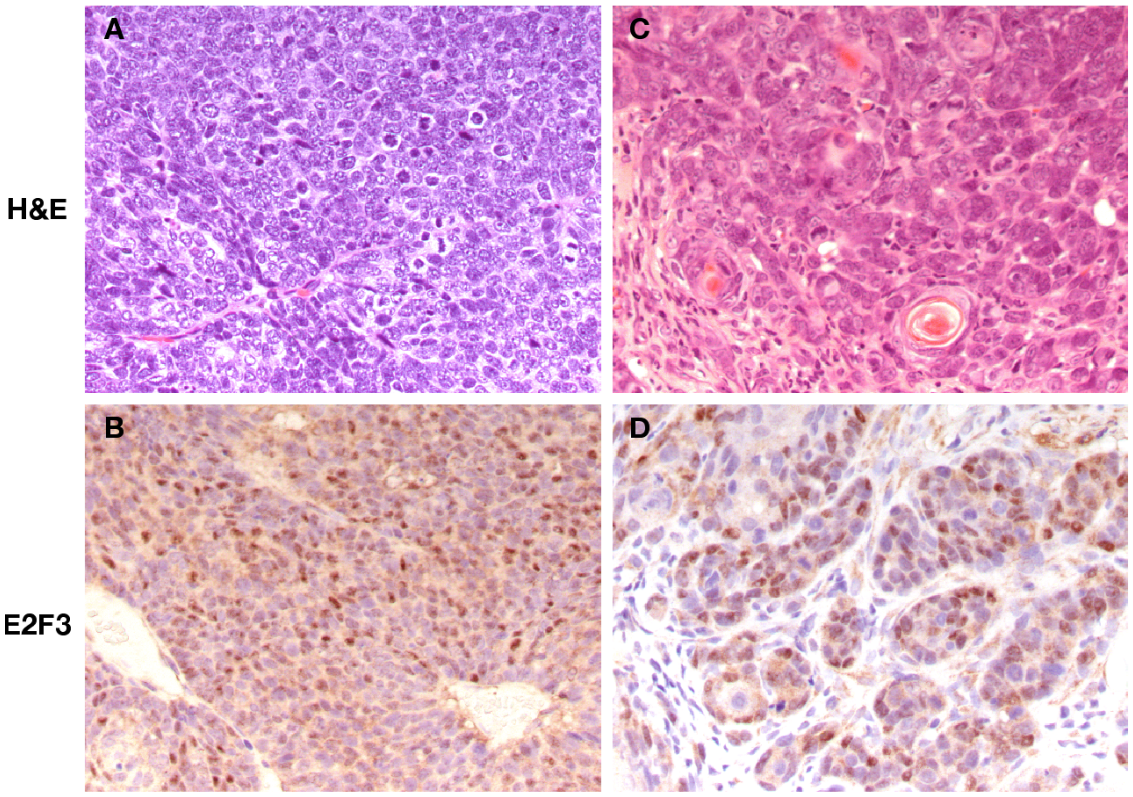
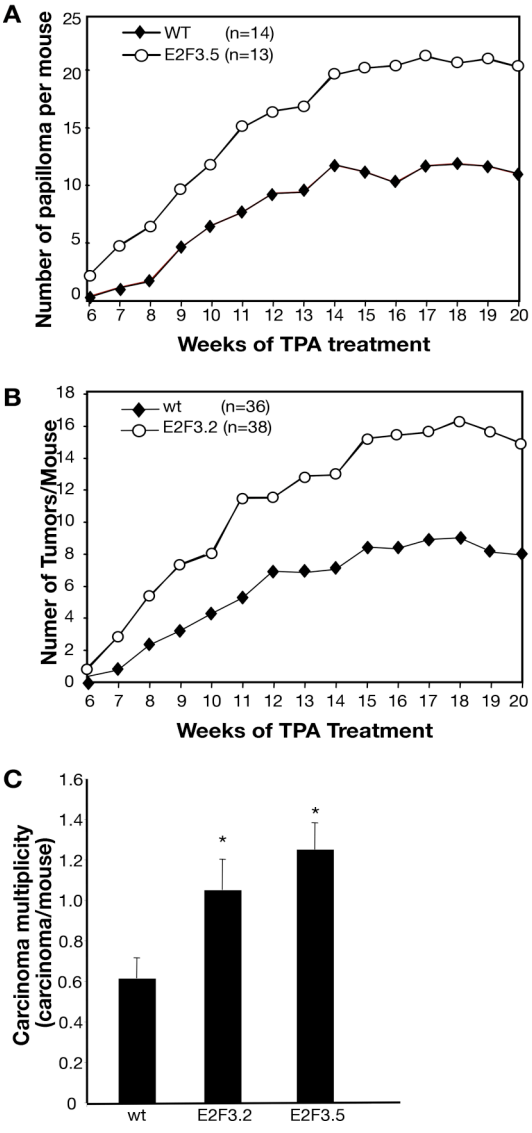


Table 1 Spontaneous K5-positive tumors in K5 E2F3a mice

line	sex	age (months)	location	Histological appearance
3.2	M	15	skin	Squamous Cell Carcinoma
3.2	M	24	skin	Papilloma
3.2	M	24	thymus	Thymoma
3.2	F	19	mammary gland	Carcinoma
3.2	F	14	salivary gland	Adenocarcinoma
3.5	F	21	vagina	Squamous Cell Carcinoma
3.5	F	14	mammary gland	Carcinoma
3.5	F	21	thymus	Thymoma
3.5	F	21	vagina	Squamous Cell Carcinoma

Figure 14. K5 E2F3a transgenic mice have an enhanced response to two-stage skin carcinogenesis.

K5 E2F3a transgenic mice from line 3.5 (A) and line 3.2 (B) along with non-transgenic sibling controls (wt) were subjected to the two-stage skin carcinogenesis assay. Skin papilloma development was recorded weekly and the average number of papillomas per mouse was determined for each group. (C) Carcinoma development was determined in the mice subjected to the two-stage carcinogenesis assay above after 30 weeks of promotion and expressed as carcinomas per mouse. Diagnosis by a veterinary pathologist confirmed that lesions were squamous cell carcinomas. Bars indicate standard error; *p <0.01 compared to wild type control group.



at two years of age was not statistically different from the incidence of their wild type siblings (Figure 12A and B). However, when tumors of only keratin 5-expressing tissues were considered, there was a clear difference between K5 E2F3a transgenic and wild type mice (Figure 12C and D). In both lines, approximately 20% of transgenic mice developed tumors in K5-expressing tissues by two years of age while none of the wild type mice developed tumors in K5-expressing tissues. All epithelial tumors from K5 E2F3a transgenic mice included in Figure 12C and D and listed in Table 1 were confirmed to express keratin 5 and overexpress E2F3 as determined by immunohistochemistry (Figure 13). The majority of tumors in wild type mice were alveolar bronchiolar adenomas, which do not express K5 and are a common background tumor in the FVB strain. Despite the fact that E2F3 is overexpressed in human bladder and prostate cancers, no tumors in these K5-expressing tissues were observed in K5 E2F3a transgenic mice.

To further address the role of E2F3a in tumorigenesis, K5 E2F3a transgenic mice and their wild type siblings were subjected to a two-stage skin carcinogenesis protocol. This carcinogenesis assay is one of the most common models for studying multistage tumor development. Initiation of carcinogenesis was carried out by a single topical application of 7, 12-dimethylbenz[a]anthracene (DMBA) to the dorsal skin of six- to eight-week old mice. DMBA induces a mutation at codon 61 of the c-Ha-*ras* gene in initiated cells. From the beginning of the third week after DMBA treatment, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was applied to the initiated area twice a week for a total of 20 weeks. This promotion process results in hyperplasia of the skin and eventually the outgrowth of exophytic papillomas. A subset of these benign papillomas can convert to malignant skin carcinomas. By 20 weeks of promotion, both lines of K5 E2F3a transgenic mice developed approximately twice as many skin papillomas as the wild type sibling control group (Figure 14A and B). The multiplicity of carcinomas at 30

weeks of promotion was slightly higher in both K5 E2F3 transgenic lines compared to wild type controls (Figure 14C). The above results suggest that E2F3a functions as an oncogene *in vivo*. When it is deregulated, E2F3a induces tumor development both spontaneously and with carcinogenic chemical stimulation.

3.3 DISCUSSION

Most human cancers have a disruption in the p16INK4a-cyclin D-RB pathway resulting in the deregulation of activator E2Fs, among them E2F3a. Several studies have shown amplification of the *E2f3* gene and/or increased E2F3 expression in human cancers including transitional cell carcinoma of the bladder and prostate cancers (Feber et al., 2004; Foster et al., 2004; Oeggerli et al., 2004). Also E2F3a is used as a marker for large-cell lung cancer (Borczuk et al., 2003). This is consistent with the finding that E2F3 overexpression can stimulate cell proliferation and contribute to oncogenic transformation *in vitro* (DeGregori et al., 1997; Xu et al., 1995). In a mouse model with *Rb*–deficient tumors, *E2f3* loss results in suppression of pituitary tumors but promotes the development and metastasis of medullary thyroid carcinomas (Ziebold et al., 2003). This suggests that E2F3 activity is a key event for tumorigenesis downstream of *Rb* loss in some tissues. At the same time, E2F3 may also have tumor suppressive activity in different *Rb* mutant tumors. In human tumors, the inactivation of *E2f3* has not been reported, so the relevance of this tumor suppressive activity for E2F3 is unclear.

The K5 E2F3a transgenic model was generated to examine the role of deregulated E2F3a in cell proliferation and apoptosis in an *in vivo* system, and more importantly, to explore the role of E2F3a in tumorigenesis. In this model, E2F3a is ectopically expressed in a number of epithelial tissues starting at day 13 of embryonic development (Ramirez et

al., 1994). In the epidermis, overexpression of E2F3a leads to hyperproliferation and hyperplasia as well as an increased level of apoptosis. In a previous study in which E2F3a is inducibly expressed in the pituitary gland, the induction of E2F3a led to hyperproliferation and hyperplasia but the sustained overexpression of E2F3a did not result in tumor formation (Denchi et al., 2005). In our K5 E2F3a transgenic model, overexpression of E2F3a did modestly induce tumor development in some epithelial tissues. Furthermore, the K5 E2F3a transgenic mice have an increased sensitivity to chemical carcinogenesis in the DMBA/TPA two-stage assay.

Among the E2F family members that have been tested in the K5 transgenic model, E2F3a as well as E2F1 induce apoptosis while E2F4 lacks this apoptosis inducing activity, yet, all three members induce hyperproliferation and hyperplasia in the epidermis. Even though both E2F3a and E2F1 induce apoptosis, E2F1 induced apoptosis is largely p53 dependent as shown by reduced levels of apoptosis after inactivation of *p53,* while the inactivation of *p53* does not affect E2F3a-induced apoptosis. This suggests that E2F3a and E2F1 utilize different mechanisms to induce apoptosis. In this K5 transgenic mouse model, E2F3a also displays different oncogenic properties than that of E2F1 and E2F4. K5 E2F3a transgenic mice develop epithelial tumors that are not observed in wild type siblings. These tumors develop late in life starting at 14 months of age and at a relatively low incidence. K5 E2F1 transgenic mice develop spontaneous epithelial tumors starting at about one year of age and these mice have a higher incidence of tumor formation (Pierce et al., 1999). On the other hand, E2F4 transgenic mice are not predisposed to developing tumors (Wang et al., 2000). It is possible that the level of E2F protein expression differs among these models and that this explains the difference in tumor development. However, we do not believe this is the case since the levels of hyperproliferation and hyperplasia are similar or higher in the E2F4 and E2F3 transgenic

models compared to K5 E2F1 transgenic mice (Bartkova et al., 2005; Pierce et al., 1998a; Wang et al., 2000). As mentioned earlier, both E2F1 and E2F3 have been shown to have tumor suppressive activities. In the two-stage carcinogenesis assay, K5 E2F1 transgenic mice are resistant to tumor development compared to the wild type siblings. (Pierce et al., 1999). In contrast to K5 E2F1 transgenic mice, K5 E2F3a transgenic mice are more sensitive to two-stage carcinogenesis than wild type mice. While this finding further demonstrates that deregulated E2F3a activity can contribute to tumor development, it also reveals that like E2F4, E2F3a lacks the tumor suppressive activity of E2F1 in this model system.

Chapter IV Characterizing E2F3a apoptotic pathways

4.1 RATIONALE AND SIGNIFICANCE

One of the novel but established functions of E2F3a is its apoptotic activity. E2F3 has been shown to contribute to apoptosis in several tissues in *Rb* mutant embryos (Saavedra et al., 2002; Ziebold et al., 2001). Even though some overexpression studies suggested that apoptosis is a specific property of E2F1 (DeGregori et al., 1997; Kowalik et al., 1998; Lissy et al., 2000), other cell culture and recent *in vivo* mouse model studies demonstrate that ectopically expressed E2F3a induces apoptosis *in vitro* and *in vivo* (Lazzerini Denchi and Helin, 2005; Paulson et al., 2006).

In the classical E2F apoptosis pathway, deregulation of E2F1 transactivates the *Arf* tumor suppressor gene (Bates et al., 1998; Robertson and Jones, 1998). ARF interacts with Mdm2 and inhibits its ability to target p53 for degradation (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998). Thus, increased levels of ARF mediated by E2F1 results in p53 stabilization and accumulation to induce apoptosis. However, several reports demonstrate that deregulated E2F1 induces apoptosis in a p53-dependent but ARF-independent manner (Rogoff et al., 2002; Russell et al., 2002; Tolbert et al., 2002; Tsai et al., 2002). In the absence of ARF, E2F1-induced apoptosis is correlated with p53 phosphorylation by the ATM kinase (Rogoff et al., 2002; Rogoff et al., 2004). However, the mechanism of how E2F1 activates ATM is unclear. Currently, a pathway of E2F1-induced apoptosis that is mediated by ATM kinase is becoming accepted. ATM is a serine/threonine kinase that belongs to the phosphatidylinositol 3 kinase-like kinase (PIKK) family. In response to DNA double-strand breaks (DSBs), ATM directly phosphorylates p53 on serine15 (Banin et al., 1998;

Canman et al., 1998; Tibbetts et al., 1999) and indirectly promotes the phosphorylation of p53 on other residues by phosphorylating and activating additional kinases such as Chk1, Chk2 and Plk3 (Ahn et al., 2000; Gatei et al., 2003; Xie et al., 2001). Phosphorylation of p53 at N-terminal residues inhibits Mdm2 binding, increases p53 transcriptional activity and stimulates other posttranslational modifications that regulate DNA binding.

Recent reports have demonstrated that a number of oncogenic factors, such as E2F1, cyclin E, and Myc, stimulate the phosphorylation of p53 and some other ATM targets (Bartkova et al., 2005; Lindstrom and Wiman, 2003; Powers et al., 2004; Vafa et al., 2002). Consistent with these findings, the ATM DNA damage response pathway has been shown to be activated early during the formation of several types of human tumors (Bartkova et al., 2005; Bartkova et al., 2006; Gorgoulis et al., 2005). It has been speculated that the activation of this checkpoint response by oncogenic stress is a mechanism to inhibit the formation or progression of cancer. In chapter III, we demonstrated that transgenic expression of E2F3a induces spontaneous tumor formation *in vivo* and increases sensitivity to the two-stage carcinogenesis assay (Paulson et al., 2006). These findings demonstrate an oncogenic property for E2F3a. To address the relationship between the oncogenic and apoptotic activity of E2F3a, we first need to understand the mechanisms and pathways of E2F3a induced apoptosis. We show here that E2F3a-induces apoptosis by activating the ATM DNA damage response pathway. ARF and E2F1 also contribute to E2F3a induced apoptosis *in vivo* and in human cells. Taken together, E2F3a induces apoptosis through multiple pathways. Among these pathways, the loss of ATM has the strongest impact on apoptosis impairment. The interactions between the different pathways are not completely clear, but we show here that the activation of ATM by E2F3a is not dependent on either E2F1 or ARF.

4.2 RESULTS

4.2.1 Overexpression of E2F3a induces markers of DNA double-strand breaks *in vivo*

ATM kinase has been considered to be activated by genotoxic stress. However, several recent studies show that oncogenic signals also activate ATM to induce cell cycle checkpoint responses, which lead to apoptosis or senescence (Bartkova et al., 2005; Bartkova et al., 2006; Gorgoulis et al., 2005). These findings indicate that oncogenic signals and genotoxic stress might be more inter-related, and cause similar end effects on DNA to activate the ATM pathway. In chapter III we used a transgenic mouse model expressing E2F3a in squamous epithelial tissues to demonstrate that deregulated expression of E2F3a leads to hyperproliferation, apoptosis and spontaneous tumor development (Paulson et al., 2006). To determine if expression of E2F3a, as an oncogenic stress, causes DNA damage *in vivo*, we examined markers of the DNA damage response (DDR) in K5 E2F3a transgenic tissue. One of the most widely used markers of DDR is the phosphorylated form of the histone variant H2AX, known as γH2AX (Celeste et al., 2003; Lukas et al., 2003; Lukas et al., 2004; Mochan et al., 2004; Stucki and Jackson, 2004). γH2AX is induced by IR (ionizing irradiation), a potent inducer of DSBs. No γH2AX foci were observed in wild type epidermis while in IR treated epidermis, the formation of γH2AX nuclear foci were readily apparent (Figure 15). Similar to what is observed in IR treated tissue, γH2AX foci were also present in untreated K5 E2F3a transgenic epidermis.

A number of kinases have been reported to phosphorylate H2AX in response to genotoxic stress. To determine the role of the ATM kinase in E2F3a induced γH2AX

Figure 15. Deregulated E2F3a induces DNA damage marker γH2AX foci formation *in vivo*

Immunofluoresent staining was performed on skin sections using antibody specific for γH2AX. (A) Untreated wild type epidermis has undetectable γH2AX foci. (B) Obvious γH2AX foci formation in the epidermis from wild type mice exposed to 3 Gy of IR, and kept alive for 20 minutes before killing. (C) K5 E2F3a transgenic mice wild type for *Atm* has increased γH2AX foci in epidermis as IR treated mice while (D) K5 E2F3a transgenic mice null for *Atm* lack the staining.

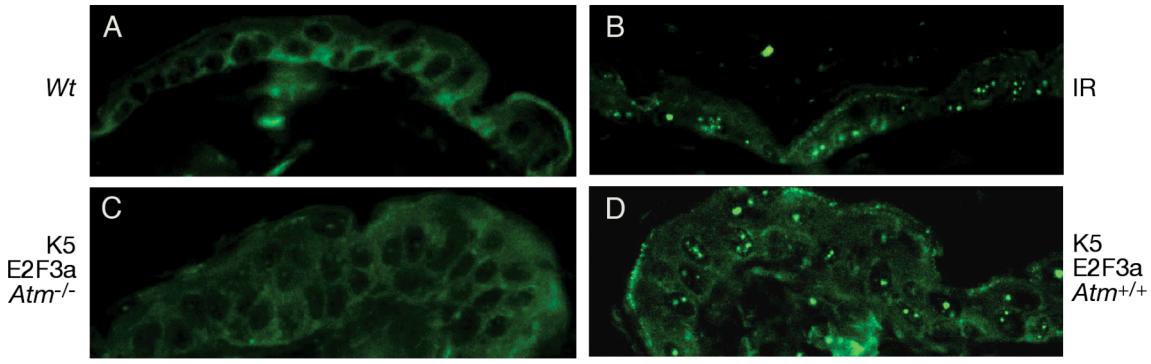
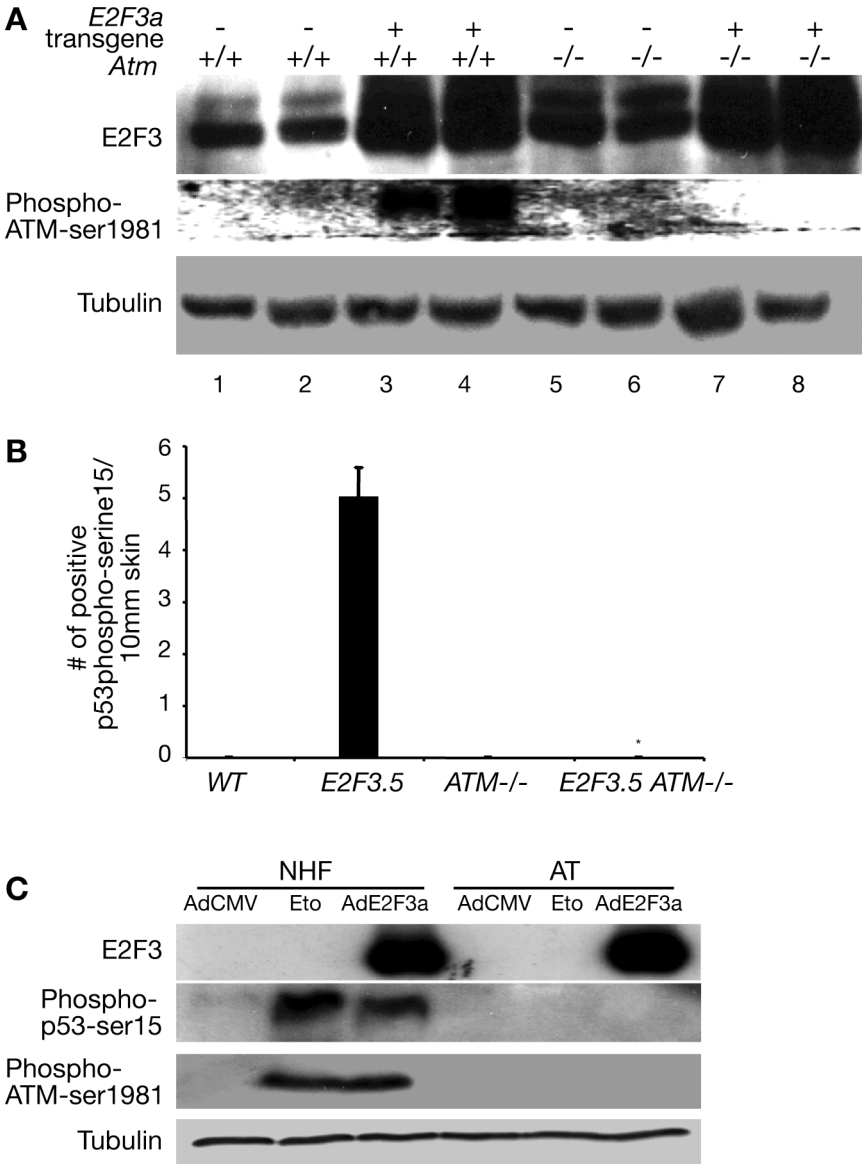


Figure 16. Overexpression of E2F3a activates ATM in K5 E2F3a transgenic mice and in human cell cultures.

(A) Western blot analysis for ATM-phospho-serine 1981 and tubulin was performed using protein extract from mice with genotypes as indicated. E2F3 expression was confirmed using E2F3 specific antisera to these protein extracts. K5 E2F3a transgenic epidermis null for *Atm* has almost undetectable levels of phospho-serine 1981 level, while K5 E2F3a transgenic epidermis wild type for *Atm* has significantly increased level of phospho-serine 1981 compared to wild type control mice. (B) Skin sections from mice with genotypes as indicated were immunochemically stained using p53-phospho-serine 15 specific antisera. Positively stained basal layer keratinocytes were calculated microscopically. K5 E2F3a transgenic mice null for *Atm* had much reduced level of p53-phospho-serine 15 positive cells compared to K5 E2F3a transgneic mice wild type for *Atm* (*$p < 0.01$). (C) Cell lysates were extracted from NHFs and AT cells that were infected with control adenovirus (AdCMV) and E2F3a expressing adenovirus (Ad E2F3a). Etoposide (Eto), a DNA damage-inducing agent was used as a positive control. AdE2F3a infected NHFs has elevated levels of ATM-phospho-serine 1981 and p53-phospho serine 15 while AT cells lack both.

Fig. 16


A
E2F3a transgene
Atm
- +/+
- +/+
+ +/+
+ +/+
- -/-
- -/-
+ -/-
+ -/-
E2F3
Phospho-ATM-ser1981
Tubulin
1 2 3 4 5 6 7 8
B
# of positive p53phospho-serine15/ 10mm skin
0 1 2 3 4 5 6
WT
E2F3.5
ATM-/-
E2F3.5 ATM-/-
C
NHF
AT
AdCMV Eto AdE2F3a AdCMV Eto AdE2F3a
E2F3
Phospho-p53-ser15
Phospho-ATM-ser1981
Tubulin

foci formation, K5 E2F3a transgenic mice were crossed into an *Atm*$^{-/-}$ background. In the absence of ATM, γH2AX immunostaining was much reduced in E2F3a transgenic epidermis. This suggests that when overexpressed, E2F3a signals to ATM directly or indirectly, and the activated ATM mediates the phosphorylation of H2AX in response to deregulated E2F3a (Figure 15).

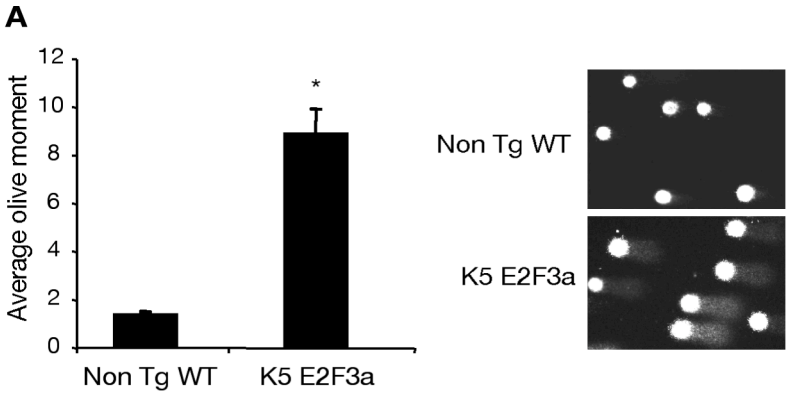
Another well established marker of DSB and ATM activation is autophosphorylation of ATM at serine 1981 (Bakkenist and Kastan, 2003). To further confirm activation of ATM by overexpression of E2F3a and determine the mechanism of E2F3a induced apoptosis, western blot analysis was performed using mouse epidermal lysates and a phospho-specific antibody to ATM to demonstrate that transgenic expression of E2F3a did indeed lead to ATM phosphorylation at serine 1981 (Figure 16A). We also examined phosphorylation of p53 at serine 15, a direct target of ATM kinase, in E2F3a transgenic tissue by immunohistochemistry. Consistent with the finding that E2F3a induces the phosphorylation of ATM at serine 1981, K5 E2F3a transgenic epidermis showed increased staining for phospho-p53 at serine15 in the basal layer of the epidermis and hair follicles compared to wild type mice (Figure 16B). Inactivation of *Atm* in E2F3a transgenic mice reduced the level of phospho-p53 staining to background levels. In normal human fibroblasts (NHFs), overexpression of E2F3a also induced the autophosphorylation of ATM at serine 1981 and the phosphorylation of p53 at serine 15. This response was similar to the response observed in NHFs treated with the DNA damaging drug etoposide. As in transgenic tissue, the phosphorylation of p53 in response to E2F3a expression was dependent on ATM since it did not occur in primary fibroblasts isolated from an AT patient, which lack functional ATM (Figure 16C). Taken together, these findings demonstrate that E2F3a overexpression in K5 E2F3a transgenic mice and

in human cells leads to the activation of ATM and the phosphorylation of downstream targets H2AX and p53.

4.2.2 Deregulated expression of E2F3a causes DNA damage.

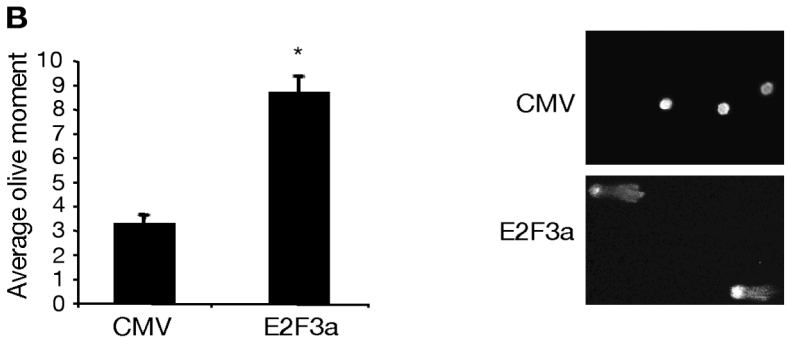
The ATM kinase plays a pivotal role in the immediate response of cells to DSBs but it can also respond to other stresses that do not involve DNA damage. To determine if deregulated E2F3a activity causes DNA damage as the mechanism of activating ATM, we used the single cell gel electrophoresis (comet) assay. Relatively few primary keratinocytes isolated from wild type mice exhibited comet tails, indicating little DNA damage in these cells. In contrast, primary keratinocytes isolated from K5 E2F3a transgenic mice showed a much-increased percentage of cells with visible comet tails. Calculations of the average olive tail moment, a measure of both the amount and distribution of DNA in the tail, confirmed that E2F3a transgenic keratinocytes had significantly increased DNA damage compared to wild type keratinocytes (Figure 17A). To confirm and extend this finding to human cells, we overexpressed E2F3a in NHFs using recombinant adenovirus expressing E2F3a and performed the comet assay. Consistent with the results from the transgenic keratinocytes, NHFs infected with E2F3a expressing adenovirus exhibited increased levels of DNA damage compared to NHFs infected with a control adenovirus vector (Figure 17B). Our laboratory has also shown that deregulation of endogenous E2F3 contributes to DNA damage (unpublished data). Specifically, expression of the E1A oncoprotein, which binds and inactivates RB, significantly increased levels of DNA damage compared to control cells. When E1A expressing cells are transfected with small interfering RNA (siRNA) specific for E2F3 to

Figure 17. Deregulated E2F3a induces DNA damage in primary keratinocytes and human cell cultures as measured by the comet assay.
(A) Primary keratinocytes obtained from the epidermis of newborn K5 E2F3a transgenic mice and sibling wild type mice were subjected to the comet assay. The percentage cells with tails were counted microscopically and the average tail moment was analyzed using CometScore software from TriTek. (B) Normal human fibroblasts were infected with control and E2F3a expressing adenovirus. Post-24 hours of infection, cells were subjected to the comet assay and analyzed as above.



Cells	% with tails	Average olive moment
WT	9.7	1.4 ± 0.1
K5 E2F3a	46.3	8.9 ± 1.0*

*,p < 0.001, as compared with wild type samples.



Cells	% with tails	Average olive moment
CMV	11.4	3.3 ± 0.5
E2F3a	42.6	8.6 ± 1.1*

*,p < 0.001, as compared with control cells.

knock down E2F3 expression, the level of DNA damage observed in E1A expressing cells was reduced while a control siRNA had no effect.

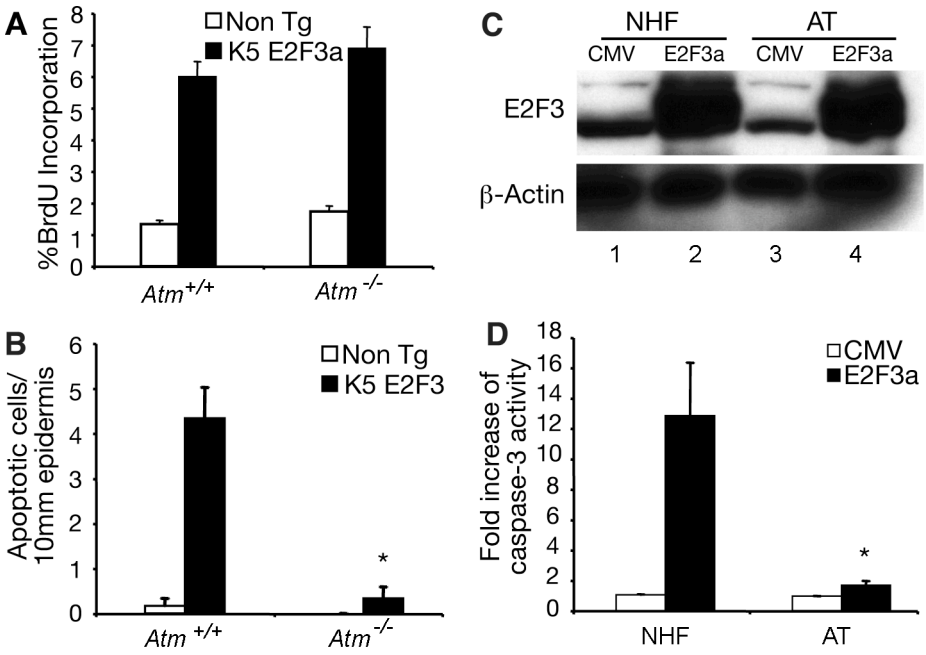
4.2.3 E2F3a-induced apoptosis is dependent on ATM in K5 E2F3a transgenic mice and in human cell cultures.

To establish the relevance of the ATM DNA damage response pathway to E2F3a induced apoptosis, tissue from wild type and K5 E2F3a transgenic mice homozygous or nullizygous for *Atm* were examined. Epidermal cells expressing the activated form of caspase 3 were detected in skin samples by immunohistochemistry as a measure of apoptosis. As previous shown (Paulson et al., 2006), the number of caspase 3 positive cells in K5 E2F3a transgenic epidermis is significantly increased over the level seen in wild type sibling epidermis (Figure 18B). This increase in the level of apoptosis was largely abolished in transgenic epidermis when *Atm* was inactivated, while hyperproliferation was not significantly affected by the absence of ATM (Figure 18A and B). The importance of ATM in E2F3a induced apoptosis was also tested in primary human cell cultures. NHFs and AT fibroblasts were infected with a control adenovirus vector or with adenovirus expressing E2F3a. As in transgenic epidermis, E2F3a overexpression induced a significantly increased level of apoptosis in NHFs while AT fibroblasts were relatively resistant to the apoptotic effects of E2F3a (Figure 18 C and D). We conclude that E2F3a induced apoptosis is largely dependent on ATM both *in vivo* and *in vitro*.

Figure 18. Inactivation of *Atm* reduces apoptosis in K5 E2F3a transgenic mice and human cell cultures.

(A) Skin sections taken from mice with the indicated genotypes were immunohistochemically stained for BrdU incorporation. The percentage of interfollicular basal layer keratinocytes staining for BrdU was calculated microscopically for each sample. At least 1000 basal layer cells from three or four different sections of the same skin sample were calculated. The average from five independent mice in each genotype is presented. The loss of ATM does not affect the BrdU incorporation level induced by E2F3a. (B) Skin sections from mice as above were immunohistochemically stained with an antibody specific for the activated form of caspase-3. The average number of positive epidermal interfollicular basal layer cells per 10 mm of skin was determined microscopically. The reduction in the number of caspase-3 positive cells in K5 E2F3a transgenic mice null for *Atm* is statistically significant compared to the number in K5 E2F3a trangenic mice wild type for *Atm* (*$p < 0.01$). (C) Western blot analysis of protein lysates from NHF and AT cells infected with E2F3a expressing adenovirus (E2F3a) (lane 2 and 4) and control virus (CMV) (lane 1 and 3) using antisera specific for E2F3 and β-actin. (D) Caspase-3 activity assay performed on cell lysates from NHF and AT cells. Each genotype of cells were infected with control adenovirus (CMV) or E2F3a expressing adenovirus (E2F3a). Caspase-3 activity was measured by fluorescence intensity of digested caspase-3 substrate AFC-DEVD using FL600 fluorescence reader. The fold increase of caspase-3 activity in E2F3a overexpressing AT cells were significantly reduced compared to E2F3a overexpressing NHFs (*$p < 0.01$).

Fig. 18


A
%BrdU Incorporation
Non Tg
K5 E2F3a
Atm+/+
Atm-/-
B
Apoptotic cells/ 10mm epidermis
Non Tg
K5 E2F3
Atm+/+
Atm-/-
C
NHF
AT
CMV
E2F3a
CMV
E2F3a
E2F3
β-Actin
1
2
3
4
D
Fold increase of caspase-3 activity
CMV
E2F3a
NHF
AT

4.2.4 Replication inhibition reduces E2F3a induced DNA damage and ATM mediated apoptosis

Several mechanisms have been suggested to contribute to DNA damage response, including ROS accumulation (Calviello et al., 2006; Vafa et al., 2002), telomere attrition (d'Adda di Fagagna et al., 2003; Reaper et al., 2004) and aberrant replication induced replication stress (Bartkova et al., 2005; Bartkova et al., 2006; Gorgoulis et al., 2005). To determine if E2F3a-induced DNA damage and following responses involve aberrant DNA replication, we used the DNA polymerase α inhibitor aphidicolin to inhibit DNA replication. Indeed, the DNA damage measured by the comet assay revealed that in the presence of aphidicolin, E2F3a induced much shorter comet tails and less DNA damage represented by decreased olive moment (Figure 19). In eukaryotic cells, Cdk activity is required for initiation of DNA replication. After assembly of the pre-initiation replication complex (pre-RC), Cdk phosphorylates the pre-RC component Cdc6 and ORC to recruit all the DNA replication proteins to the replication fork (Brown et al., 1997; Elsasser et al., 1996) and promotes the origin firing and DNA synthesis. To explore whether Cdk activity was involved in DNA damage and apoptosis as a result of E2F3a overexpression, the p21 Cdk inhibitor was co-expressed in NHFs. Continued overexpression of p21 significantly reduced E2F3a-induced DNA damage (Figure 20A). The activation of the ATM signalling pathway represented by ATM autophosphorylation at serine 1981 and the phosphorylation of its down stream targets p53 at serine 15 and γH2AX are also impaired (Figure 20B). Significantly, apoptosis in response to E2F3a is decreased in the presence of overexpressed p21 as well (Figure 20C).

Figure 19. DNA replication inhibitor Aphidicolin reduces E2F3a-induced DNA damage.

NHFs were infected with a recombinant adenovirus expressing control vector, or E2F3a. At the same time, cells were treated with DNA replication inhibitor Aphidicolin (aph) at 5μg/ml. Cells were subjected to the comet assay post-24 hours of treatments. DNA damage was measured by olive moment which was analyzed as in Figure 17. Aphidicolin treatment significantly reduced DNA damage induced by E2F3a overexpression.

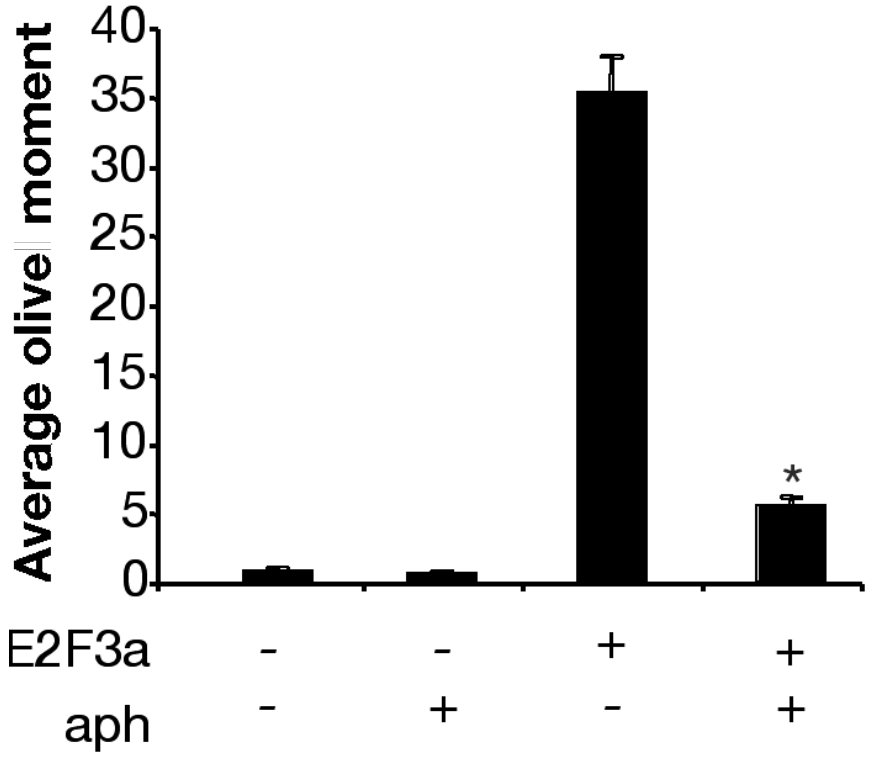
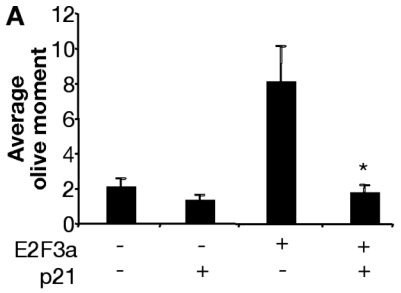
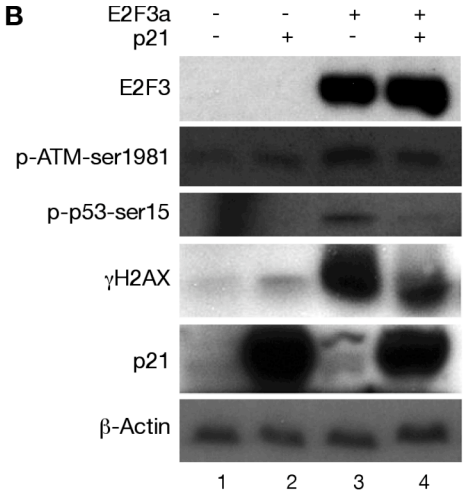


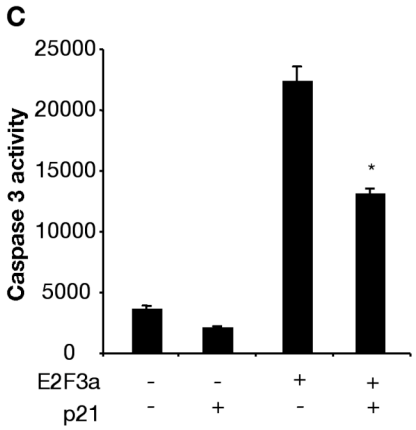
Figure 20. Overexpression of Cdk inhibitor p21 reduces E2F3a-induced DNA damage and response.

(A) NHFs infected with recombinant adenovirus expressing control vector, p21, E2F3a and E2F3a plus p21. 24 hours post-infection, cells were subjected to the comet assay. DNA damage is represented by olive tail moment. (B) Cell lysates were extracted from cells infected with above adenoviruses post-48 hours of infection. Immunoblot analysis was carried out using antibodies specific for phospho-ATM serine 1981, phospho-p53 serine 15 and γH2AX. β-actin serves as a loading control. (C) Whole cell lysates from cells treated as above were subjected to caspase 3 activity assay.



Cells	% with tails	Average olive moment
Control	10.1	2.2 ± 0.4
p21	7.2	1.4 ± 0.3
E2F3a	48.3	8.2 ± 2.0
E2F3a + p21	25.9	1.8 ± 0.4*





4.2.5 E2F3a activates ATM independent of E2F1 or p19^{ARF}.

It has been shown that the *E2f1* gene promoter region contains E2F DNA-binding sites and that it is an established downstream target of E2F transcriptional activity (Johnson et al., 1994). Furthermore, several E2Fs including E2F3 induces E2F1 expression by binding to the E2F sites in the promoter region of the *E2f1* gene and activating its promoter (Araki et al., 2003; Neuman et al., 1994; Wells et al., 2000). This inter-regulating ability of E2F family members may explain the phenomenon that the E2F transcription factors have their specific yet sometimes overlapping roles in cell proliferation, apoptosis, differentiation and development.

Previous studies have suggested that among the E2F family, E2F1 is a unique inducer of apoptosis. In fact, in cases where E2F3a induces apoptosis, it has been suggested that this occurs through the E2F3a-mediated activation of E2F1 (Lazzerini Denchi and Helin, 2005). We and others have demonstrated that E2F1-induced apoptosis is also dependent on ATM (Powers et al., 2004; Rogoff et al., 2004), although the mechanism of how E2F1 activates ATM is not clear. Nonetheless, these findings suggest the possibility that E2F3a could activate ATM and induce apoptosis through the upregulation of E2F1.

To determine whether E2F3a could activate ATM through the regulation of E2F1, we first confirmed that overexpression of E2F3a upregulates endogenous E2F1 expression at the protein level in both the E2F3a transgenic mouse model (Figure 21A) and in normal human fibroblast cultures infected with a recombinant adenovirus expressing E2F3a (Figure 21B). These findings are consistent with the fact that the *E2f1* gene promoter contains E2F DNA binding sites and that it is an established downstream

Figure 21. Overexpression of E2F3a upregulates endogenous E2F1 protein level.

(A) Western blot analysis of protein extract isolated from the epidermis of K5 E2F3a transgenic mice line3.2 (E2F3.2, lane 2 and 3), line 3.5 (E2F3.5, lane 4 and 5) and wild type sibling mice (wt, lane1) using antisera specific for E2F3, E2F1 or β-actin. (B) Primary human fibroblasts, NHF (Normal Human Fibroblast) and AT (Fibroblasts from AT patients with non-functional ATM) were infected with E2F3a expressing adenovirus (lane 3 and 6) and control virus (lane 1 and 4). Etoposide (Eto) treated NHF and AT cells served as a DNA damage treatment control (lane 2 and 5). Protein lysates from the above treated cells were subjected to western blotting analysis with antisera specific for E2F3, E2F1 or β-actin.

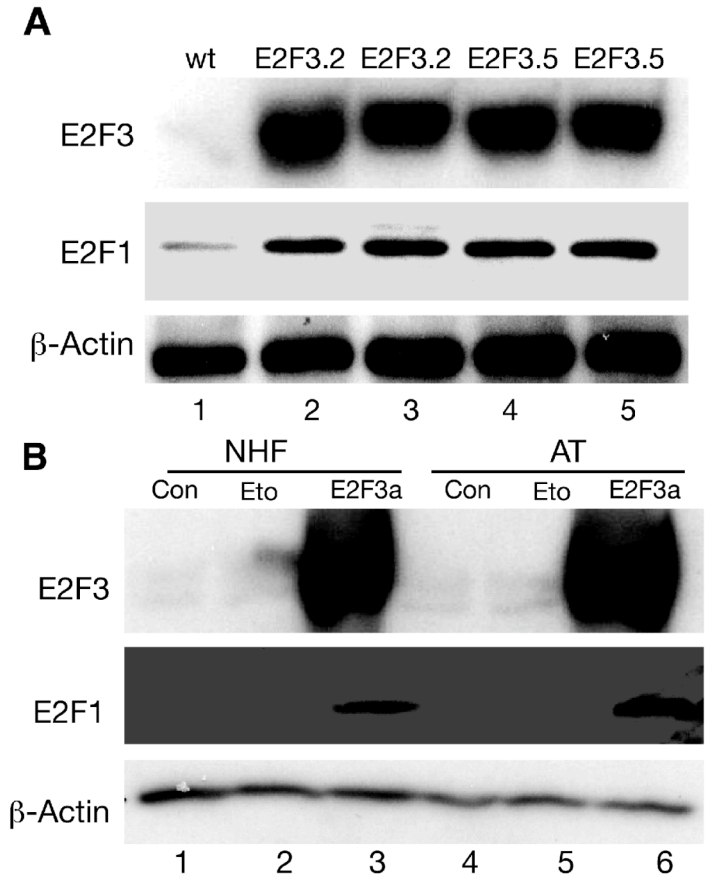


Figure 22. E2F3a induces E2F1-dependent apoptosis *in vivo* and *in vitro.*

(A) Skin sections taken from mice with the indicated genotypes were immunohistochemically stained for BrdU incorporation. The percentage of interfollicular basal layer keratinocytes staining for BrdU was calculated microscopically for each sample. The average from five independent mice in each genotype is presented. (B) Skin sections from mice as above were immunohistochemically stained with an antibody specific for the activated form of caspase 3. The average number of positive epidermal interfollicular basal layer cells per 10 mm of skin was determined microscopically. The number of caspase 3 positive cells in K5 E2F3a transgenic mice null for *E2f1* is significantly reduced compared to the number in K5 E2F3a trangenic mice wild type for *E2f1* (*$p < 0.01$). (C) Western blot analysis of protein lysates from MAFs, with genotype as indicated, infected with E2F3a expressing adenovirus (E2F3a) (lane 2 and 4) and control virus (CMV) (lane 1 and 3) using antisera specific for E2F3 and β-actin. (D) Caspase-3 activity assay performed on cell lysates from MEFs with genotypes as indicated. Each genotype of cells were infected with control adenovirus (CMV) or E2F3a expressing adenovirus (E2F3a). Caspase 3 activity was determined by measuring digested caspase-3 substrate AFC-DEVD fluorescence intensity in each sample using FL600 fluorescence reader. The fold increase in caspase-3 activity in E2F3a overexpressing cells null for *E2f1* was significantly reduced compared to E2F3a overexpressing cells wild type for *E2f1* (*$p < 0.01$).

Fig. 22

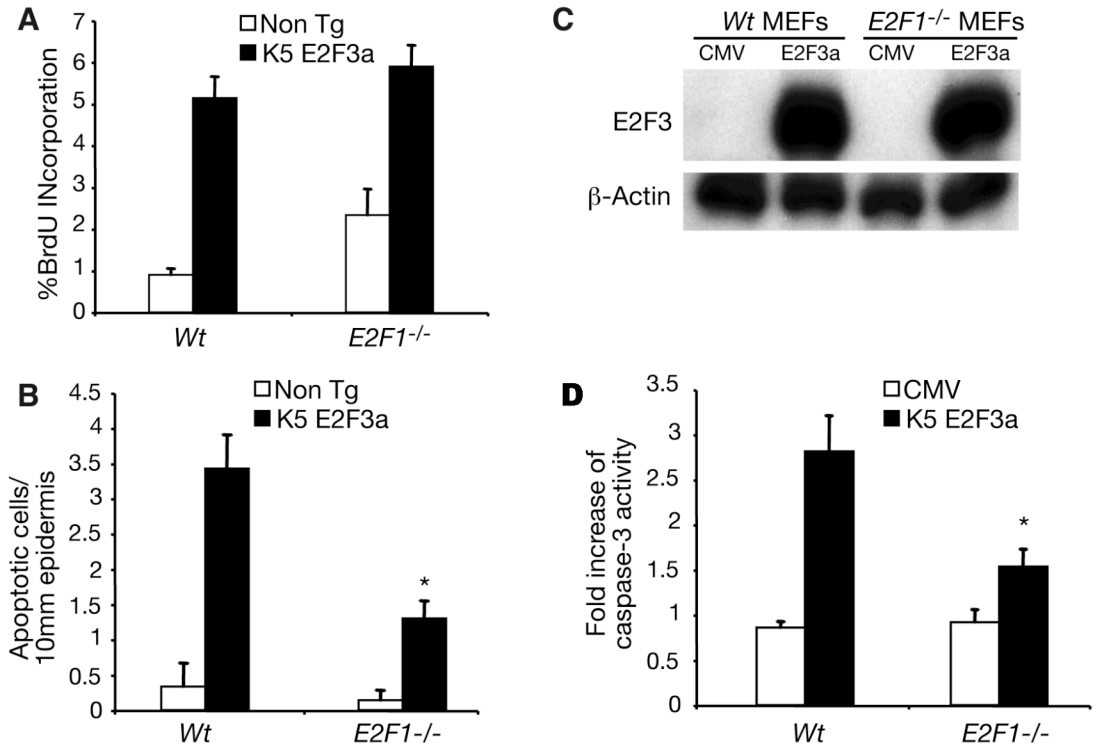

A
%BrdU INcorporation
Non Tg
K5 E2F3a
Wt
E2F1-/-
B
Apoptotic cells/ 10mm epidermis
Non Tg
K5 E2F3a
*
Wt
E2F1-/-
C
Wt MEFs
E2F1-/- MEFs
CMV
E2F3a
CMV
E2F3a
E2F3
β-Actin
D
Fold increase of caspase-3 activity
CMV
K5 E2F3a
*
Wt
E2F1-/-

Figure 23. E2F3a activates ATM independent of E2F1.
(A) MEFs with genotype as indicated were infected with control virus (CMV) and adenovirus expressing E2F3a (E2F3a). IR irradiation is used as DNA damage positive control. Cell lysates from above treated cells were subjected to western blot analysis with specific antisera to E2F3, E2F1, phospho-p53 serine 15 and phospho-ATM serine 1981. Actin serves as loading control. E2F3a overexpressing cells wild type or null for *E2f1* showed little difference in the level of phospho-ATM and phospho-p53. (B) γH2AX foci formation shown by immunofluoresent staining using antibody specific for γH2AX. Skin sections were obtained from mice with genotype as indicated. IR irradiated skin section serves as a positive control. The loss of E2F1 did not affect E2F3a induced γH2AX foci formation.

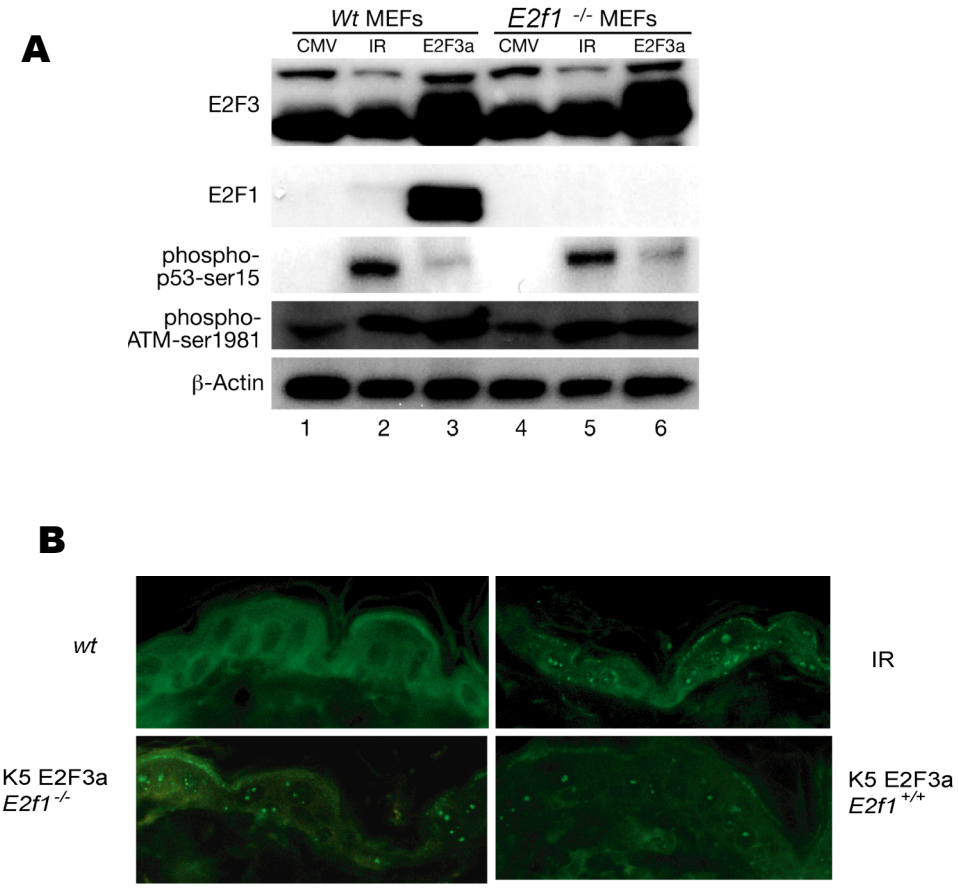
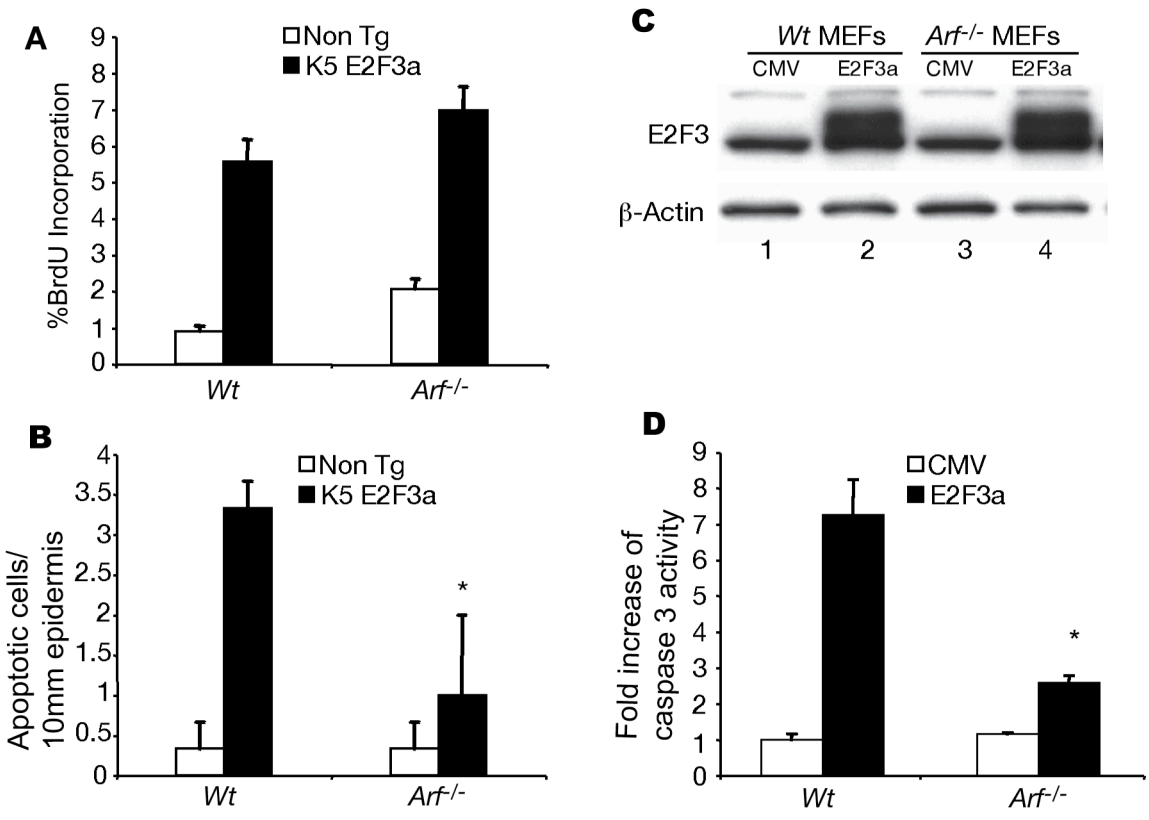


Figure 24. Inactivation of *Arf* reduces apoptosis *in vivo and in vitro.*

(A) Skin sections taken from mice with the indicated genotypes were immunohistochemically stained for BrdU incorporation. The percentage of interfollicular basal layer keratinocytes staining for BrdU was calculated microscopically for each sample. At least 1000 basal layer cells from three or four different sections of the same skin were calculated. The average from five independent mice in each genotype is presented. (B) Skin sections from mice as above were immunohistochemically stained with an antibody specific for the activated form of caspase-3. The average number of positive epidermal interfollicular basal layer cells per 10 mm of skin was determined microscopically. The reduction in the number of caspase-3 positive cells in K5 E2F3a transgenic mice null for *Arf* is statistically significant compared to the number in K5 E2F3a transgenic mice wild type for *Arf* (*$p < 0.01$). (C) Western blot analysis of protein lysates from MEFs, with genotype as indicated, infected with E2F3a expressing adenovirus (E2F3a) (lane 2 and 4) and control virus (CMV) (lane 1 and 3) using antisera specific for E2F3 and β-actin. (D) Caspase-3 activity assay performed on cell lysates from MEFs with genotypes as indicated. Each genotype of cells were infected with control adenovirus (CMV) or E2F3a expressing adenovirus (E2F3a). Caspase-3 activity was determined by measuring fluorescence intensity of digested caspase-3 substrate AFC-DEVD in each sample using FL600 fluorescence reader. The fold increase of caspase-3 activity in E2F3a overexpressing cells null for *E2f1* were significantly impaired compared to E2F3a overexpressing cells wild type for *Arf* (*$p < 0.01$).

Fig. 24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9
Wt
Arf-/-
B
Non Tg
K5 E2F3a
Apoptotic cells/ 10mm epidermis
0
0.5
1
1.5
2
2.5
3
3.5
4
*
Wt
Arf-/-
C
Wt MEFs
Arf-/- MEFs
CMV
E2F3a
CMV
E2F3a
E2F3
β-Actin
1
2
3
4
D
CMV
E2F3a
Fold increase of caspase 3 activity
0
1
2
3
4
5
6
7
8
9
*
Wt
Arf-/-

target of E2F transcriptional activity (Araki et al., 2003; Neuman et al., 1994; Wells et al., 2000).

To further address the role of E2F1 in E2F3a induced apoptosis, we crossed K5 E2F3a transgenic mice and wild type siblings into an *E2f1*$^{-/-}$ background. Inactivation of *E2f1* did not affect the ability of E2F3a to stimulate cell proliferation as indicated by similar levels of BrdU incorporation in K5 E2F3a transgenic epidermis in the presence or absence of E2F1 (Figure 22A). In contrast, inactivation of *E2f1* significantly reduced the level of apoptosis observed in K5 E2F3a transgenic epidermis (Figure 22B). To confirm these findings in a cell culture system, primary mouse embryonic fibroblasts (MEFs) isolated from wild type and *E2f1*$^{-/-}$ mice were infected with either a control virus or one containing E2F3a. As in epidermial tissue, inactivation of *E2f1* reduced the level of apoptosis induced by E2F3a in these cultured cells (Figure 22C and D).

To determine if the E2F1 dependency of E2F3a-induced apoptosis is connected to ATM pathway activation, we examined ATM serine 1981 and p53 serine 15 phosphorylation in wild type and *E2f1*$^{-/-}$ MEFs infected with adenovirus expressing E2F3a. MEFs treated with IR served as a positive control for ATM pathway activation. Western blot analysis showed no difference in ATM or p53 phosphorylation between wild type and *E2f1*$^{-/-}$ cells overexpressing E2F3a (Figure 23A). Consistent with those findings, γH2AX foci formation in the epidermis of K5 E2F3a epidermis was unaffected when *E2f1* was inactivated (Figure 23B). In summary, these findings suggest that while E2F1 participates in E2F3a-induced apoptosis, E2F1 is dispensable for ATM pathway activation in response to E2F3a.

ARF stabilizes and activates p53 by interacting with and inhibiting the p53 inhibitor Mdm2 E3 ubiquitin ligase (Kamijo et al., 1998; Zhang et al., 1998). Newer findings suggest that E2F1 induces ARF-independent ATM-dependent apoptosis (Russell

et al., 2002; Tolbert et al., 2002; Tsai et al., 2002). The ATM kinase and tumor suppressor ARF play major roles in tumor surveillance. ARF is induced by hyperproliferative signals emanated from oncogenes, this induction of ARF will eventually lead to cell cycle arrest and apoptosis or senescence. Recently multiple studies have shown that oncogenic signals also activate ATM checkpoint responses to induce apoptosis or senescence (Bartkova et al., 2005; Bartkova et al., 2006; Gorgoulis et al., 2005). This suggests that genotoxic stress and oncogenic signals are more tightly interrelated than initially thought.

The ARF tumor suppressor is a transcriptional target of E2F3 and we have shown that ARF expression is upregulated in K5 E2F3a transgenic mice (Paulson et al., 2006). It has been suggested that ARF contributes to ATM activation (Aslanian et al., 2004). To examine the role of ARF in E2F3a-induced apoptosis and ATM activation, K5 E2F3a transgenic mice were crossed into an *Arf* null background. Apoptosis in K5 E2F3a transgenic epidermis was reduced by *Arf* inactivation while hyperproliferation was unaffected by the absence of ARF (Figure 24A and B). A similar defect in E2F3a-induced apoptosis was observed in $Arf^{-/-}$ MEFs infected with an adenovirus encoding E2F3a (Figure 24C and D). In summary, even though E2F3a induced apoptosis is p53-independent, E2F3a-induced apoptosis is impaired in the absence of ARF.

Having established that E2F3a-induced apoptosis is partially dependent upon ARF in both *in vivo* and *in vitro* systems, we next examined whether ARF was involved in ATM activation in response to E2F3a. Wild type and $Arf^{-/-}$ MEFs were infected with control or E2F3a expressing recombinant adenovirus and ATM autophosphorylation was examined as a marker of ATM pathway activation. No difference in ATM autophosphorylation was observed between wild type and $Arf^{-/-}$ cells overexpressing E2F3a (Figure 25). Taken together, these findings demonstrate that E2F3a requires

Figure 25. E2F3a activates ATM independent of ARF.

Wild type and *Arf*$^{-/-}$ MEFs obtained from sibling embryos were infected with control adenovirus (CMV) or adenovirus expressing E2F3a (E2F3a). Cell lysates were subjected to western blot analysis using antisera to E2F3, phospho-ATM-serine1981 and actin. No obvious difference in the level of phospho-ATM in E2F3a overexpressing cells null for *Arf* (lane 6) compared to E2F3a overexpressing cells wild type for *Arf* (lane3).

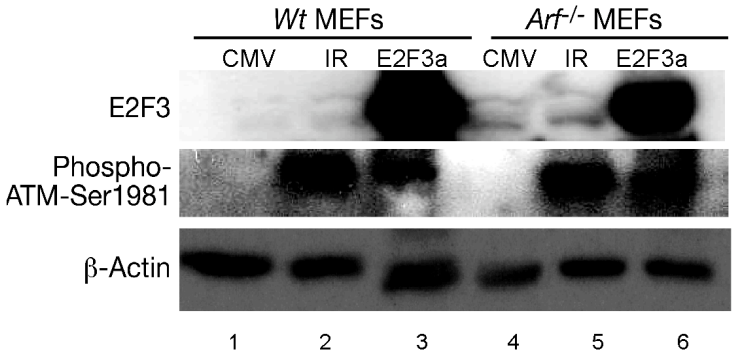


Figure 26. Expression of p19^{ARF} increases in K5 E2F3a transgenic epidermis with wild type or *E2f1* null background.

Whole RNA was extracted from mouse epidermis with genotype as indicated. Real time PCR was performed on samples obtained from at least three mice from each genotype. The relative expression value was normalized to *GAPDH* expression. In both wild type and *E2f1*$^{-/-}$ MEFs, E2F3a overexpression induced similar folds of increase of ARF expression compared to controls, but *E2f1* inactivation reduced both basal level and E2F3a-induced level of ARF expression.

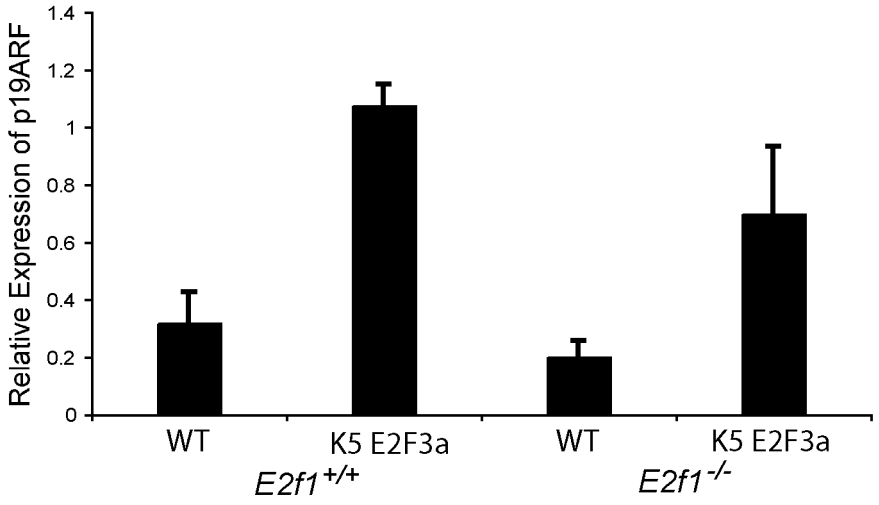
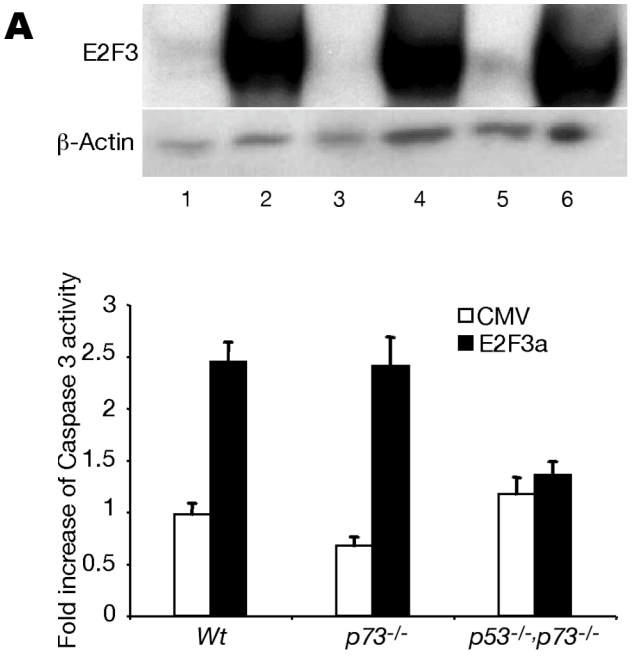
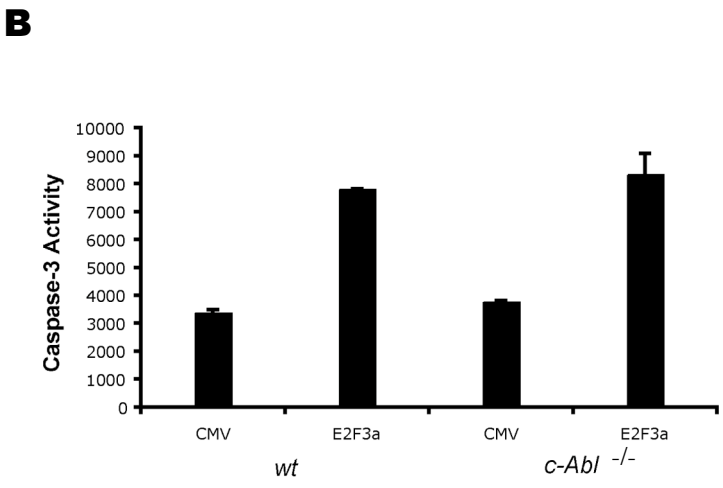


Figure 27. Loss of both p53 and p73 impairs E2F3a-induced apoptosis.
(A) MEFs with genotypes as indicated were infected with recombinant adenovirus expressing E2F3a or control virus. Whole cell lysates was extracted 48 hours post infection. A caspase 3 activity assay was carried out with AFC-DEVD as a substrate of caspase 3. The activity is represented by the intensity of fluorescence. (B) Wild type and *c-Abl* null MEFs were extracted from the same litter embryos. Both genotypes of MEFs were infected with control and E2F3a expressing adenovirus and caspase 3 activity measured as above.





ATM, E2F1 and ARF for the efficient induction for apoptosis in both transgenic mouse epidermis and in primary fibroblasts cultures but that E2F1 and ARF are not involved in ATM pathway activation in response to E2F3a overexpression. Further, K5 E2F3a upregulates ARF expression level to the same extent in the presence or absence of E2F1 (Figure 26). This further indicates that E2F3a utilizes a complicated mechanism that involves several parallel pathways instead of a linear apoptosis pathway (Figure 28).

4.2.6 E2F3a induced apoptosis requires either p53 or p73

We and others have previously demonstrated that E2F3a-induced apoptosis *in vivo* is unaffected by the absence of p53 (Figure 11) (Lazzerini Denchi and Helin, 2005; Paulson et al., 2006). This leaves an apparent paradox since it is believed that p53 is a critical mediator of apoptosis downstream of ATM, ARF and E2F1. This raises the question of how E2F3a-induced apoptosis can be independent of p53 but dependent on several upstream regulators of p53. Previous studies have suggested several factors that may be responsible for ATM, ARF or E2F1 to bypass p53 to induce apoptosis. Among them, the p53 related protein p73 has been shown to promote apoptosis in response to several stresses (Gong et al., 1999; Jost et al., 1997; Stiewe and Putzer, 2000; Yuan et al., 1999). To examine the role of p73 in E2F3a-induced apoptosis, $p73^{-/-}$ mouse embryo fibroblasts (a kind gift from Dr. Elsa Flores at UT M.D.Anderson Cancer Center) were infected with E2F3a expressing adenovirus or a control virus. As with the absence of p53, the absence of p73 also did not affect apoptosis induced by E2F3a overexpression (Figure 27A). Consistent with this finding, the absence of the p73 regulating kinase c-Abl is also dispensable for E2F3a-induced apoptosis (Figure 27B). However, in MEFs deficient for

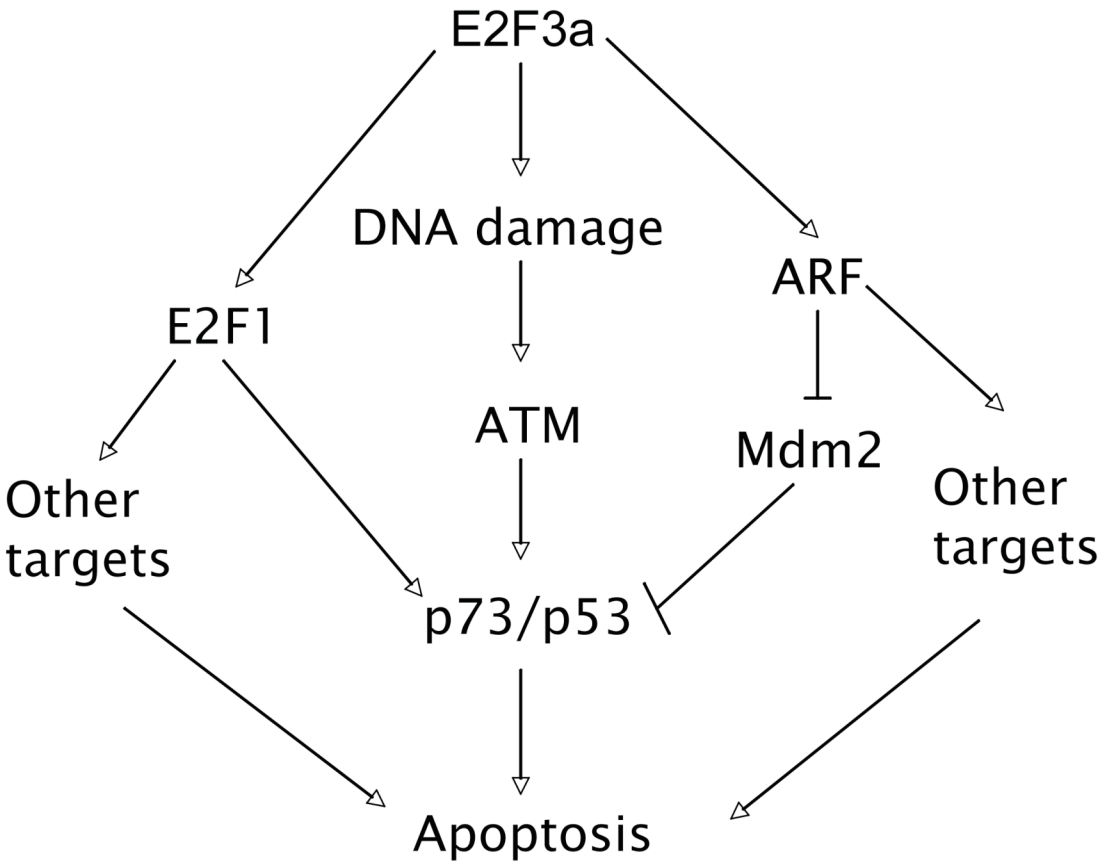
both p53 and p73 (a kind gift from Dr. Elsa Flores at UT M.D. Anderson Cancer Center), apoptosis in response to E2F3a was much decreased (Figure 27A). This indicates that p53 and p73 may compensate for each other in promoting E2F3a-induced apoptosis.

4.3 DISCUSSION

The induction of apoptosis is crucial for elimination of cells with deregulated proliferation and serves as an important barrier for tumorigenesis. In the E2F family, E2F1 has been extensively studied for its role in apoptosis and has been considered to be the specific E2F member that induces apoptosis (DeGregori et al., 1997; Kowalik et al., 1998; Lissy et al., 2000). There's evidence that in primary mouse fibroblast culture systems, ectopic expression of E2F3 induces apoptosis (Lazzerini Denchi and Helin, 2005). Along with others, we show in an *in vivo* system that E2F3a overexpression induces apoptosis (Lazzerini Denchi and Helin, 2005; Paulson et al., 2006). Our finding in human fibroblast cultures further demonstrates that ectopic expression of E2F3a induces apoptosis in a primary human system.

While E2F3a induces apoptosis *in vitro* and *in vivo*, the mechanism by which E2F3a induces apoptosis is largely unknown. In the classical pathway of E2F-induced apoptosis, deregulated E2F transcriptional activity upregulates ARF expression. ARF inhibits the function of Mdm2, which leads to p53 stabilization and accumulation. Upregulated p53 in turn transcribes proapoptotic mitochondria proteins and induces apoptosis. An earlier study suggested that E2F3-induced apoptosis is E2F1 dependent (Lazzerini Denchi and Helin, 2005). In this study, in both K5 E2F3a transgenic mice and

Figure 28. Schematic model of E2F3a-induced apoptosis pathways.



cell cultures, we show that overexpression of E2F3a upregulates E2F1 expression and that the loss of E2F1 impairs the ability of E2F3a to induce apoptosis. While E2F3a mediated apoptosis requires E2F1, there's also E2F1-independent pathways by which E2F3a promotes apoptosis. Several studies have shown that ARF is dispensable in E2F1-induced apoptosis, on the contrary, we show that inactivation of *Arf* reduces E2F3a-induced apoptosis. While E2F3a upregulates E2F1 and ARF levels and induces E2F1- and ARF-dependent apoptosis, our data show that the loss of E2F1 does not affect the upregulation of ARF expression induced by E2F3a. This suggests that even though E2F1 is required for E2F3a-induced apoptosis, E2F3a has a distinct apoptotic induction pathway from E2F1 that involves ARF but not p53.

Several studies have shown that overexpression of oncogenic growth factors, cyclin E, Cdc25, or E2F1 in cultured cells can activate the ATM signaling pathway (Bartkova et al., 2005; Lindstrom and Wiman, 2003; Powers et al., 2004; Vafa et al., 2002). Consistent with those findings, we find that E2F3a overexpression can also cause DNA damage and activate ATM. Furthermore, when ATM function is lost, it largely reduces E2F3a-induced apoptosis. Even though both E2F1 and ARF have been reported to activate ATM and promote p53 dependent apoptosis (Li et al., 2004; Pauklin et al., 2005; Powers et al., 2004), E2F3a-induced ATM activation is not impaired in either *Arf* or *E2f1* deficient MEFs. E2F3a-induced γH2AX foci formation in mouse epidermis is also not affected by the loss of E2F1, which suggests that the E2F3a-induced DNA damage response pathway is independent of E2F1. In summary, these data suggest that in E2F3a-induced apoptosis, instead of one inter-related pathway with different factors regulating each other, there are several parallel pathways. Also note that eliminating a key factor does not completely diminish apoptosis induced by E2F3a, but rather only

reduces the level of apoptosis. This suggests that the three pathways cooperate and may partially compensate for each other's function (Figure 28).

One interesting phenomenon we showed here (Paulson et al., 2006) and also been shown by another group (Lazzerini Denchi and Helin, 2005) is that deregulated E2F3a induces p53-independent apoptosis. E2F3a-induced apoptosis is partially E2F1 dependent, and E2F1 induces both p53-dependent and p53-independent apoptosis. In E2F1 induced p53-independent apoptosis, the p53 homologue p73 is believed to be directly transcriptionally activated by E2F1, leading to activation of p53 responsive genes and apoptosis (Irwin et al., 2000; Stiewe and Putzer, 2000). While it is widely accepted that ATM and ARF stabilize p53 and this leads to apoptosis, there are also evidences that ATM and ARF also regulate p73 to induce apoptosis (Agami et al., 1999; Gong et al., 1999; Wang et al., 2001; Yuan et al., 1999). Using p73 knockout MEFs, we did not see apoptosis being affected by loss of p73, but when p53 and p73 were both absent, E2F3a-induced apoptosis was significantly reduced. This provides evidence that p53 and p73 both contribute to E2F3a-induced apoptosis but suggests that p53 and p73 functionally compensate for each other in apoptosis induction by deregulated E2F3a.

Besides p73, all three factors E2F1, ATM and ARF have been shown to regulate proapoptotic proteins including Bcl-2 family proteins, Apaf-1 or effector caspases such as caspase 3, 7 or 6. DNA microarray studies show that ectopic expression of E2Fs up-regulates expression of proapoptotic members of the Bcl-2 family, such as Bok, Bad, Bak and Bid1, and effector caspases, including caspase 3 and caspase 7 (Ma et al., 2002; Muller et al., 2001). Induction of E2F1 activity also results in an increase of mRNA and protein levels of Apaf1, which activates caspase 9 and consequently caspase 3 and caspase 6 (Moroni et al., 2001). ARF may also induce apoptosis independently of p53 (Weber et al., 2000), by inducing Bax (Suzuki et al., 2003). ATM has recently been

shown to phosphorylate proapoptotic Bcl-2 family member Bid in response to DNA damage, suggesting a possible mechanism for ATM-induced p53-independent apoptosis (Kamer et al., 2005; Zinkel et al., 2005). These proapoptotic factors are potential targets for E2F3a-induced, p53-independent apoptosis.

Chapter V Concluding remarks

5.1 SUMMARY AND DISCUSION OF THE STUDIES

In this dissertation, I present several novel findings, with one being that we provide the first evidence showing that E2F3a, when deregulated, functions as an oncogene and contributes to tumorigenesis in a transgenic mouse model. We also show that unlike E2F1, deregulated E2F3a does not suppress, but enhances DMBA/TPA two-stage skin carcinogenesis. Second, we demonstrate that the overexpression of E2F3a can induce apoptosis *in vivo* and *in vitro*, in mouse and human cells. Thus, in the E2F family, E2F3a and E2F1 both have apoptotic induction activity. Third, E2F3a induces p53-independent apoptosis and further findings show that the p53 family member p73 and p53 compensate for each other's function in E2F3a induced-apoptosis. Fourth, E2F3a induces DNA damage and this appears to involve increased Cdk activity and aberrant DNA replication. Fifth, the ATM DNA damage pathway is activated in response to E2F3a induced-DNA damage and this is important for apoptosis. Finally, we show that E2F3a induces apoptosis through unique pathways by activating several parallel pathways involving ATM, E2F1 and ARF. I will expand on these findings in the following disccusions.

So far, K5 transgenic mouse models for E2F1, E2F3a and E2F4 have now been developed in our laboratory (Pierce et al., 1998a; Pierce et al., 1998b; Wang et al., 2000). This allows a comparison of the in vivo activities of these E2F family members in the same model system with the caveat that the level of exogenous E2F proteins may not be exactly the same (Table 2). Each of the E2F family members tested induces hyperproliferation and hyperplasia in the epidermis of transgenic mice. E2F3a and E2F1

also induce increased levels of apoptosis while E2F4 lacks an apoptosis induction activity. However, the mechanism by which E2F3a and E2F1 induce apoptosis appears to be different since *p53* inactivation reduces apoptosis in K5 E2F1 transgenic mice but not in K5 E2F3a transgenic mice. This agrees with findings from Denchi and Helin demonstrating that apoptosis induced by transgenic expression of E2F3 in the pituitary gland is also independent of p53 (Lazzerini Denchi and Helin, 2005). Paradoxically, our further findings show that E2F3a-induced apoptosis is dependent on E2F1. The loss of E2F1 significantly impairs the increased levels of apoptosis induced by E2F3a overexpression. We speculate that E2F3a, while it does not require p53 to induce apoptosis, does require other proapoptotic activities of E2F1. Another unique property of E2F3a-induced apoptosis is that ARF is required but not p53. This indicates that ARF has other activities that are independent of p53.

In the effort of finding the mechanistic pathway of E2F3a-induced p53-independent apoptosis, we tested the role of the p53 family member p73. As one of the factors that are important in p53-independent apoptosis induced by E2F1, p73 is a downstream target of E2F1 and the activation of p73 has been shown to provide a means for E2F1 to induce cell death in the absence of p53 (Irwin et al., 2000). Furthermore, the disruption of *p73* function inhibits E2F1-induced apoptosis in *p53* null cells (Lissy et al., 2000). ARF also regulates p73 through inhibition of Mdm2 and results in upregulation of p73 and its transcriptional activity (Wang et al., 2001). It was found that like p53, p73 is also induced by DNA damage in a c-Abl-dependent manner (Agami et al., 1999; Gong et al., 1999; Yuan et al., 1999). This induction of p73 is mediated through the interaction between the SH3 domain of c-Abl and the C-terminal homo-oligomerization domain of p73, and the phosphorylation of p73 on Tyrosine 99 by c-Abl. Consistent with the result that loss of *c-Abl* does not affect E2F3a-induced apoptosis, in p73 deficient MEFs, the

Table 2 Comparison of K5 E2F Transgenic models

Increased Proliferation	K5 transgene	Increased Apoptosis	Spontaneous Tumorigenesis	Effect on Skin Carcinogenesis
+	E2F1	+ (p53-dependent)	+	↓
+	E2F3	+ (p53-independent)	+/-	↑
+	E2F4	-	-	↑

increase in E2F3a-induced apoptosis is unaffected compared to wild type MEFs overexpressing E2F3a. However, in MEFs deficient for both p53 and p73, we see a significant decrease in E2F3a-induced apoptosis compared to wild type MEFs. This leads us to conclude that either p53 or p73 is dispensable in E2F3a-induced apoptosis but that they compensate for each other's function when one of them is absent and the loss of both p53 and p73 largely impairs E2F3a-induced apoptosis. To further address the regulation of p73 by E2F3a, we have shown that the upregulation of p73 transcription by E2F3a is not significant. Post-translational phosphorylation of p73 could provide another mechanism by which E2F3a regulates p73.

Together with earlier findings that E2F3a is a strong regulator of cell proliferation, the finding that deregulated E2F3a induces hyperproliferation and tumor development in the K5 E2F3a transgenic model confirmed the oncogenic property of the *E2f3a* gene. Recent studies have indicated the existence of tumorigenesis barriers that involve oncogene-induced DNA damage, which leads to activation of the ATM DNA damage response pathway and thereby to apoptosis or senescence (Bartkova et al., 2005; Bartkova et al., 2006; Di Micco et al., 2006; Gorgoulis et al., 2005). In this study, we show that deregulated E2F3a expression results in the accumulation of DNA damage, most likely double-strand breaks. This was shown by γH2AX foci formation, a DSB damage marker, in both K5 E2F3a transgenic mouse epidermis and normal human fibroblasts infected with a recombinant adenovirus expressing E2F3a. In a more direct approach, we used the comet assay to show that deregulated E2F3a induces DNA damage in both the K5 E2F3a transgenic keratinocytes and normal human fibroblasts. In response to E2F3a-induced DNA damage, the checkpoint kinase ATM is activated, shown by increased autophosphorylation at serine 1981, a well-established marker of

ATM activation and DSB. This activation of ATM contributes to apoptosis induced by E2F3a. In K5 E2F3a transgenic mice, inactivation of *Atm* significantly reduces the level of apoptosis compared to K5 E2F3a transgenic mice with wild type *Atm*. To explore the mechanism of E2F3a-induced DNA damage, we show using the comet assay that the DNA replication inhibitior aphidicolin largely reduces DNA damage caused by deregulated E2F3a. Furthermore, in all eukaryotes, Cdks drive the major cell cycle events of DNA replication and mitosis (Forsburg and Nurse, 1991; Reed, 1992). We show in this study that the Cdk inhibitor p21, when ectopically expressed in human cells, significantly reduces E2F3a-induced DNA damage. At the same time, co-expression of p21 also reduces the activation of ATM and the level of apoptosis induced by deregulated E2F3a. These results provide a possible mechanism for E2F3a induced DNA damage response, which can be summarized as aberrant DNA replication promoted by deregulated E2F3a causes DNA damage. In response to this DNA damage, ATM signaling pathway is activated and results in the cell fate of apoptosis.

We also show that E2F3a induces apoptosis through unique pathways. E2F3a upregulates another E2F family member E2F1, and the absence of E2F1 reduces E2F3a-induced apoptosis. This indicates that E2F3a induces apoptosis partially through E2F1. Even though E2F3a induced-apoptosis is partially dependent on E2F1, it also uses a different pathway than E2F1 in apoptosis. In this pathway, E2F3a upregulates ARF and induces ARF-dependent but p53-independent apoptosis. We further show that the upregulation of ARF by E2F3a is independent of E2F1. Meanwhile, to examine how the E2F1 and ARF-dependent pathways relate to the ATM pathway, we found that the loss of either ARF or E2F1 does not affect the activation of ATM by E2F3a. Therefore, instead of a linear pathway, deregulated E2F3a activates several parallel pathways that result in the cell fate of apoptosis.

5.2 FUTURE DIRECTIONS

In this study we have convincingly shown that deregulated E2F3a induces p53-independent apoptosis through several parallel pathways and provided evidence that p73 may play a role in E2F3a-induced apoptosis. There are several proapoptotic factors that are potential targets for E2F3a-induced p53-independent apoptosis that could be addressed in future studies in order to understand the mechanism of E2F3a-indcued apoptosis even better. Besides p73, proapoptotic Bcl-2 family proteins, Apaf-1 and effector caspases such as caspase 3, 7 and 6 have been shown to be regulated by E2F1, ATM and/or ARF. DNA microarray studies show that ectopic expression of E2Fs up-regulates expression of proapoptotic members of the Bcl-2 family, such as Bok, Bad, Bak and Bid1, and effector caspases, including caspase 3 and caspase 7 (Ma et al., 2002; Muller et al., 2001). Induction of E2F1 activity also results in an increase of mRNA and protein levels of Apaf1, which when induced, activate caspase 9 and consequently caspase 3 and caspase 6 (Moroni et al., 2001). ARF can also induce apoptosis independently of p53 (Weber et al., 2000) through Bax (Suzuki et al., 2003). ATM has recently been shown to phosphorylate the proapoptotic Bcl-2 family member Bid in response to DNA damage, suggesting a possible mechanism for ATM-induced, p53-independent apoptosis (Kamer et al., 2005; Zinkel et al., 2005).

While E2F3a functions as an oncogene, when deregulated it induces tumor development, the tumors develop late in life and less than half of the mice develop a tumor. One possible explanation for this could be that E2F3a-induced apoptosis functions as a barrier for tumor development. Further experiments to characterize the

E2F3a apoptotic pathways and examine the role of E2F3a-induced apoptosis in tumor development will help us understand more fully the role of E2F3a in tumorigenesis.

Another interesting finding made during these studies is that E2F3a activates the ATM DNA damage response pathway. Recently, publications by Bartkova et al, Di Micco et al and Mallette et al have proposed that the DNA damage signaling pathway is a critical mediator of oncogene-induced senescence (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). Characterization of the role of E2F3a in senescence could also help us better understand the functions carried out by E2F3a.

References

Adams, M. R., Sears, R., Nuckolls, F., Leone, G., and Nevins, J. R. (2000). Complex transcriptional regulatory mechanisms control expression of the E2F3 locus. Mol Cell Biol *20*, 3633-3639.

Agami, R., Blandino, G., Oren, M., and Shaul, Y. (1999). Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis. Nature *399*, 809-813.

Ahn, J. Y., Schwarz, J. K., Piwnica-Worms, H., and Canman, C. E. (2000). Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res *60*, 5934-5936.

Alonso, L., and Fuchs, E. (2003). Stem cells of the skin epithelium. Proc Natl Acad Sci U S A *100 Suppl 1*, 11830-11835.

Araki, K., Nakajima, Y., Eto, K., and Ikeda, M. A. (2003). Distinct recruitment of E2F family members to specific E2F-binding sites mediates activation and repression of the E2F1 promoter. Oncogene *22*, 7632-7641.

Asano, M., Nevins, J. R., and Wharton, R. P. (1996). Ectopic E2F expression induces S phase and apoptosis in Drosophila imaginal discs. Genes Dev *10*, 1422-1432.

Aslanian, A., Iaquinta, P. J., Verona, R., and Lees, J. A. (2004). Repression of the Arf tumor suppressor by E2F3 is required for normal cell cycle kinetics. Genes Dev *18*, 1413-1422.

Bagchi, S., Raychaudhuri, P., and Nevins, J. R. (1990). Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. Cell *62*, 659-669.

Bagchi, S., Weinmann, R., and Raychaudhuri, P. (1991). The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. Cell *65*, 1063-1072.

Bakkenist, C. J., and Kastan, M. B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499-506.

Balmain, A., and Brown, K. (1988). Oncogene activation in chemical carcinogenesis. Adv Cancer Res *51*, 147-182.

Bandara, L. R., and La Thangue, N. B. (1991). Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature *351*, 494-497.

Bandara, L. R., Lam, E. W., Sorensen, T. S., Zamanian, M., Girling, R., and La Thangue, N. B. (1994). DP-1: a cell cycle-regulated and phosphorylated component of transcription factor DRTF1/E2F which is functionally important for recognition by pRb and the adenovirus E4 orf 6/7 protein. Embo J *13*, 3104-3114.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674-1677.

Barrandon, Y., and Green, H. (1987). Three clonal types of keratinocyte with different capacities for multiplication. Proc Natl Acad Sci U S A *84*, 2302-2306.

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J. M., Lukas, C., *et al.* (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature *434*, 864-870.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V., Kolettas, E., Niforou, K., Zoumpourlis, V. C., *et al.* (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998). p14ARF links the tumour suppressors RB and p53. Nature *395*, 124-125.

Bikle, D. D., Ng, D., Tu, C. L., Oda, Y., and Xie, Z. (2001). Calcium- and vitamin D-regulated keratinocyte differentiation. Mol Cell Endocrinol *177*, 161-171.

Borczuk, A. C., Gorenstein, L., Walter, K. L., Assaad, A. A., Wang, L., and Powell, C. A. (2003). Non-small-cell lung cancer molecular signatures recapitulate lung developmental pathways. Am J Pathol *163*, 1949-1960.

Botz, J., Zerfass-Thome, K., Spitkovsky, D., Delius, H., Vogt, B., Eilers, M., Hatzigeorgiou, A., and Jansen-Durr, P. (1996). Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. Mol Cell Biol *16*, 3401-3409.

Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature *391*, 597-601.

Brown, G. W., Jallepalli, P. V., Huneycutt, B. J., and Kelly, T. J. (1997). Interaction of the S phase regulator cdc18 with cyclin-dependent kinase in fission yeast. Proc Natl Acad Sci U S A *94*, 6142-6147.

Calviello, G., Piccioni, E., Boninsegna, A., Tedesco, B., Maggiano, N., Serini, S., Wolf, F. I., and Palozza, P. (2006). DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: involvement of the oxidative mechanism. Toxicol Appl Pharmacol *211*, 87-96.

Campanero, M. R., and Flemington, E. K. (1997). Regulation of E2F through ubiquitin-proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. Proc Natl Acad Sci U S A *94*, 2221-2226.

Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677-1679.

Celeste, A., Fernandez-Capetillo, O., Kruhlak, M. J., Pilch, D. R., Staudt, D. W., Lee, A., Bonner, R. F., Bonner, W. M., and Nussenzweig, A. (2003). Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol *5*, 675-679.

Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbacid, M., *et al.* (2005). Tumour biology: senescence in premalignant tumours. Nature *436*, 642.

Conti, C. J. (1994). *The Mouse Skin as a Model for Chemical Carcinogenesis.*, In Handbook of Mouse Mutations With Skin and Hair Abnormalities., S. JP, ed. (Boca Raton: CRC), pp. 39-46.

Cotsarelis, G., Sun, T. T., and Lavker, R. M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell *61*, 1329-1337.

d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature *426*, 194-198.

de Bruin, A., Maiti, B., Jakoi, L., Timmers, C., Buerki, R., and Leone, G. (2003). Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem *278*, 42041-42049.

DeGregori, J., and Johnson, D. G. (2006). Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. Curr Mol Med *6*, 739-748.

DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. Proc Natl Acad Sci U S A *94*, 7245-7250.

Denchi, E. L., Attwooll, C., Pasini, D., and Helin, K. (2005). Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. Mol Cell Biol *25*, 2660-2672.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P. G., Bensimon, A., *et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

DiGiovanni, J. (1992). Multistage carcinogenesis in mouse skin. Pharmacol Ther *54*, 63-128.

DiGiovanni, J. (1994). *Carcinogenesis Target Organ Toxicology Series*. (New York: Raven Press).

DiTullio, R. A., Jr., Mochan, T. A., Venere, M., Bartkova, J., Sehested, M., Bartek, J., and Halazonetis, T. D. (2002). 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. Nat Cell Biol *4*, 998-1002.

Du, W., Xie, J. E., and Dyson, N. (1996). Ectopic expression of dE2F and dDP induces cell proliferation and death in the Drosophila eye. Embo J *15*, 3684-3692.

Dynlacht, B. D., Moberg, K., Lees, J. A., Harlow, E., and Zhu, L. (1997). Specific regulation of E2F family members by cyclin-dependent kinases. Mol Cell Biol *17*, 3867-3875.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. Genes Dev *12*, 2245-2262.

Dyson, N., Howley, P. M., Munger, K., and Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science *243*, 934-937.

Elsasser, S., Lou, F., Wang, B., Campbell, J. L., and Jong, A. (1996). Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases. Mol Biol Cell *7*, 1723-1735.

Ewen, M. E., Xing, Y. G., Lawrence, J. B., and Livingston, D. M. (1991). Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell *66*, 1155-1164.

Feber, A., Clark, J., Goodwin, G., Dodson, A. R., Smith, P. H., Fletcher, A., Edwards, S., Flohr, P., Falconer, A., Roe, T., *et al.* (2004). Amplification and overexpression of E2F3 in human bladder cancer. Oncogene *23*, 1627-1630.

Forsburg, S. L., and Nurse, P. (1991). Cell cycle regulation in the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. Annu Rev Cell Biol *7*, 227-256.

Foster, C. S., Falconer, A., Dodson, A. R., Norman, A. R., Dennis, N., Fletcher, A., Southgate, C., Dowe, A., Dearnaley, D., Jhavar, S., *et al.* (2004). Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome. Oncogene *23*, 5871-5879.

Gandarillas, A. (2000). Epidermal differentiation, apoptosis, and senescence: common pathways? Exp Gerontol *35*, 53-62.

Gatei, M., Sloper, K., Sorensen, C., Syljuasen, R., Falck, J., Hobson, K., Savage, K., Lukas, J., Zhou, B. B., Bartek, J., and Khanna, K. K. (2003). Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. J Biol Chem *278*, 14806-14811.

Girling, R., Partridge, J. F., Bandara, L. R., Burden, N., Totty, N. F., Hsuan, J. J., and La Thangue, N. B. (1993). A new component of the transcription factor DRTF1/E2F. Nature *362*, 83-87.

Gniadecki, R. (1997). Effects of 1,25-dihydroxyvitamin D3 and its 20-epi analogues (MC 1288, MC 1301, KH 1060), on clonal keratinocyte growth: evidence for differentiation of keratinocyte stem cells and analysis of the modulatory effects of cytokines. Br J Pharmacol *120*, 1119-1127.

Gong, J. G., Costanzo, A., Yang, H. Q., Melino, G., Kaelin, W. G., Jr., Levrero, M., and Wang, J. Y. (1999). The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature *399*, 806-809.

Gorgoulis, V. G., Vassiliou, L. V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., Ditullio, R. A., Jr., Kastrinakis, N. G., Levy, B., *et al.* (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature *434*, 907-913.

Hallstrom, T. C., and Nevins, J. R. (2003). Specificity in the activation and control of transcription factor E2F-dependent apoptosis. Proc Natl Acad Sci U S A *100*, 10848-10853.

Hateboer, G., Kerkhoven, R. M., Shvarts, A., Bernards, R., and Beijersbergen, R. L. (1996). Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. Genes Dev *10*, 2960-2970.

Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F. Mol Cell Biol *18*, 6679-6697.

He, Y., Armanious, M. K., Thomas, M. J., and Cress, W. D. (2000). Identification of E2F-3B, an alternative form of E2F-3 lacking a conserved N-terminal region. Oncogene *19*, 3422-3433.

Helin, K. (1998). Regulation of cell proliferation by the E2F transcription factors. Curr Opin Genet Dev *8*, 28-35.

Helin, K., Lees, J. A., Vidal, M., Dyson, N., Harlow, E., and Fattaey, A. (1992). A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell *70*, 337-350.

Hofmann, F., Martelli, F., Livingston, D. M., and Wang, Z. (1996). The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. Genes Dev *10*, 2949-2959.

Holmberg, C., Helin, K., Sehested, M., and Karlstrom, O. (1998). E2F-1-induced p53-independent apoptosis in transgenic mice. Oncogene *17*, 143-155.

Hsieh, J. K., Fredersdorf, S., Kouzarides, T., Martin, K., and Lu, X. (1997). E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. Genes Dev *11*, 1840-1852.

Humbert, P. O., Verona, R., Trimarchi, J. M., Rogers, C., Dandapani, S., and Lees, J. A. (2000). E2f3 is critical for normal cellular proliferation. Genes Dev *14*, 690-703.

Irwin, M., Marin, M. C., Phillips, A. C., Seelan, R. S., Smith, D. I., Liu, W., Flores, E. R., Tsai, K. Y., Jacks, T., Vousden, K. H., and Kaelin, W. G., Jr. (2000). Role for the p53 homologue p73 in E2F-1-induced apoptosis. Nature *407*, 645-648.

Ishida, S., Huang, E., Zuzan, H., Spang, R., Leone, G., West, M., and Nevins, J. R. (2001). Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Mol Cell Biol *21*, 4684-4699.

Johnson, D. G., Cress, W. D., Jakoi, L., and Nevins, J. R. (1994a). Oncogenic capacity of the E2F1 gene. Proc Natl Acad Sci U S A *91*, 12823-12827.

Johnson, D. G., Ohtani, K., and Nevins, J. R. (1994b). Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev *8*, 1514-1525.

Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature *365*, 349-352.

Jost, C. A., Marin, M. C., and Kaelin, W. G., Jr. (1997). p73 is a simian [correction of human] p53-related protein that can induce apoptosis. Nature *389*, 191-194.

Kamer, I., Sarig, R., Zaltsman, Y., Niv, H., Oberkovitz, G., Regev, L., Haimovich, G., Lerenthal, Y., Marcellus, R. C., and Gross, A. (2005). Proapoptotic BID is an ATM effector in the DNA-damage response. Cell *122*, 593-603.

Kamijo, T., Weber, J. D., Zambetti, G., Zindy, F., Roussel, M. F., and Sherr, C. J. (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. Proc Natl Acad Sci U S A *95*, 8292-8297.

Kitagawa, M., Higashi, H., Suzuki-Takahashi, I., Segawa, K., Hanks, S. K., Taya, Y., Nishimura, S., and Okuyama, A. (1995). Phosphorylation of E2F-1 by cyclin A-cdk2. Oncogene *10*, 229-236.

Kovesdi, I., Reichel, R., and Nevins, J. R. (1986). Identification of a cellular transcription factor involved in E1A trans-activation. Cell *45*, 219-228.

Kowalik, T. F., DeGregori, J., Leone, G., Jakoi, L., and Nevins, J. R. (1998). E2F1-specific induction of apoptosis and p53 accumulation, which is blocked by Mdm2. Cell Growth Differ *9*, 113-118.

Kowalik, T. F., DeGregori, J., Schwarz, J. K., and Nevins, J. R. (1995). E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J Virol *69*, 2491-2500.

Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., Jr., and Livingston, D. M. (1994). Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell *78*, 161-172.

La Thangue, N. B., and Rigby, P. W. (1987). An adenovirus E1A-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. Cell *49*, 507-513.

Lavia, P., and Jansen-Durr, P. (1999). E2F target genes and cell-cycle checkpoint control. Bioessays *21*, 221-230.

Lazzerini Denchi, E., and Helin, K. (2005). E2F1 is crucial for E2F-dependent apoptosis. EMBO Rep *6*, 661-668.

Lees, J. A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K. (1993). The retinoblastoma protein binds to a family of E2F transcription factors. Mol Cell Biol *13*, 7813-7825.

Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R. S., and Nevins, J. R. (1998). E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. Genes Dev *12*, 2120-2130.

Leone, G., Nuckolls, F., Ishida, S., Adams, M., Sears, R., Jakoi, L., Miron, A., and Nevins, J. R. (2000). Identification of a novel E2F3 product suggests a mechanism for determining specificity of repression by Rb proteins. Mol Cell Biol *20*, 3626-3632.

Li, Y., Graham, C., Lacy, S., Duncan, A. M., and Whyte, P. (1993). The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev *7*, 2366-2377.

Li, Y., Wu, D., Chen, B., Ingram, A., He, L., Liu, L., Zhu, D., Kapoor, A., and Tang, D. (2004). ATM activity contributes to the tumor-suppressing functions of p14ARF. Oncogene *23*, 7355-7365.

Lindeman, G. J., Gaubatz, S., Livingston, D. M., and Ginsberg, D. (1997). The subcellular localization of E2F-4 is cell-cycle dependent. Proc Natl Acad Sci U S A *94*, 5095-5100.

Lindstrom, M. S., and Wiman, K. G. (2003). Myc and E2F1 induce p53 through p14ARF-independent mechanisms in human fibroblasts. Oncogene *22*, 4993-5005.

Lissy, N. A., Davis, P. K., Irwin, M., Kaelin, W. G., and Dowdy, S. F. (2000). A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. Nature *407*, 642-645.

Lowe, S. W., Cepero, E., and Evan, G. (2004). Intrinsic tumour suppression. Nature *432*, 307-315.

Lukas, C., Falck, J., Bartkova, J., Bartek, J., and Lukas, J. (2003). Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage. Nat Cell Biol *5*, 255-260.

Lukas, C., Melander, F., Stucki, M., Falck, J., Bekker-Jensen, S., Goldberg, M., Lerenthal, Y., Jackson, S. P., Bartek, J., and Lukas, J. (2004). Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. Embo J *23*, 2674-2683.

Lukas, J., Petersen, B. O., Holm, K., Bartek, J., and Helin, K. (1996). Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. Mol Cell Biol *16*, 1047-1057.

Ma, Y., Croxton, R., Moorer, R. L., Jr., and Cress, W. D. (2002). Identification of novel E2F1-regulated genes by microarray. Arch Biochem Biophys *399*, 212-224.

Magae, J., Wu, C. L., Illenye, S., Harlow, E., and Heintz, N. H. (1996). Nuclear localization of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. J Cell Sci *109 (Pt 7)*, 1717-1726.

Maiti, B., Li, J., de Bruin, A., Gordon, F., Timmers, C., Opavsky, R., Patil, K., Tuttle, J., Cleghorn, W., and Leone, G. (2005a). Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem *280*, 18211-18220.

Maiti, B., Li, J., de Bruin, A., Gordon, F., Timmers, C., Opavsky, R., Patil, K., Tuttle, J., Cleghorn, W., and Leone, G. (2005b). Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation. J Biol Chem.

Mallette, F. A., Gaumont-Leclerc, M. F., and Ferbeyre, G. (2007). The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. Genes Dev *21*, 43-48.

Mann, D. J., and Jones, N. C. (1996). E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest. Curr Biol *6*, 474-483.

Martinez-Balbas, M. A., Bauer, U. M., Nielsen, S. J., Brehm, A., and Kouzarides, T. (2000). Regulation of E2F1 activity by acetylation. Embo J *19*, 662-671.

Marzio, G., Wagener, C., Gutierrez, M. I., Cartwright, P., Helin, K., and Giacca, M. (2000). E2F family members are differentially regulated by reversible acetylation. J Biol Chem *275*, 10887-10892.

Michaloglou, C., Vredeveld, L. C., Soengas, M. S., Denoyelle, C., Kuilman, T., van der Horst, C. M., Majoor, D. M., Shay, J. W., Mooi, W. J., and Peeper, D. S. (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature *436*, 720-724.

Milton, A., Luoto, K., Ingram, L., Munro, S., Logan, N., Graham, A. L., Brummelkamp, T. R., Hijmans, E. M., Bernards, R., and La Thangue, N. B. (2006). A functionally distinct member of the DP family of E2F subunits. Oncogene *25*, 3212-3218.

Mochan, T. A., Venere, M., DiTullio, R. A., Jr., and Halazonetis, T. D. (2004). 53BP1, an activator of ATM in response to DNA damage. DNA Repair (Amst) *3*, 945-952.

Morkel, M., Wenkel, J., Bannister, A. J., Kouzarides, T., and Hagemeier, C. (1997). An E2F-like repressor of transcription. Nature *390*, 567-568.

Moroni, M. C., Hickman, E. S., Lazzerini Denchi, E., Caprara, G., Colli, E., Cecconi, F., Muller, H., and Helin, K. (2001). Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol *3*, 552-558.

Morris, E. J., and Dyson, N. J. (2001). Retinoblastoma protein partners. Adv Cancer Res *82*, 1-54.

Morris, R. J., and Potten, C. S. (1994). Slowly cycling (label-retaining) epidermal cells behave like clonogenic stem cells in vitro. Cell Prolif *27*, 279-289.

Morris, R. J., and Potten, C. S. (1999). Highly persistent label-retaining cells in the hair follicles of mice and their fate following induction of anagen. J Invest Dermatol *112*, 470-475.

Muller, H., Bracken, A. P., Vernell, R., Moroni, M. C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J. D., and Helin, K. (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev *15*, 267-285.

Muller, H., Moroni, M. C., Vigo, E., Petersen, B. O., Bartek, J., and Helin, K. (1997). Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. Mol Cell Biol *17*, 5508-5520.

Narita, M., Nunez, S., Heard, E., Narita, M., Lin, A. W., Hearn, S. A., Spector, D. L., Hannon, G. J., and Lowe, S. W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell *113*, 703-716.

Neuman, E., Flemington, E. K., Sellers, W. R., and Kaelin, W. G., Jr. (1994). Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol Cell Biol *14*, 6607-6615.

Nevins, J. R. (1992). E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science *258*, 424-429.

Nevins, J. R. (2001). The Rb/E2F pathway and cancer. Hum Mol Genet *10*, 699-703.

Oeggerli, M., Tomovska, S., Schraml, P., Calvano-Forte, D., Schafroth, S., Simon, R., Gasser, T., Mihatsch, M. J., and Sauter, G. (2004). E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer. Oncogene *23*, 5616-5623.

Palmero, I., Pantoja, C., and Serrano, M. (1998). p19ARF links the tumour suppressor p53 to Ras. Nature *395*, 125-126.

Pan, H., Yin, C., Dyson, N. J., Harlow, E., Yamasaki, L., and Van Dyke, T. (1998). Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. Mol Cell *2*, 283-292.

Pauklin, S., Kristjuhan, A., Maimets, T., and Jaks, V. (2005). ARF and ATM/ATR cooperate in p53-mediated apoptosis upon oncogenic stress. Biochem Biophys Res Commun *334*, 386-394.

Paulson, Q. X., McArthur, M. J., and Johnson, D. G. (2006). E2F3a stimulates proliferation, p53-independent apoptosis and carcinogenesis in a transgenic mouse model. Cell Cycle *5*, 184-190.

Pierce, A. M., Fisher, S. M., Conti, C. J., and Johnson, D. G. (1998a). Deregulated expression of E2F1 induces hyperplasia and cooperates with ras in skin tumor development. Oncogene *16*, 1267-1276.

Pierce, A. M., Gimenez-Conti, I. B., Schneider-Broussard, R., Martinez, L. A., Conti, C. J., and Johnson, D. G. (1998b). Increased E2F1 activity induces skin tumors in mice heterozygous and nullizygous for p53. Proc Natl Acad Sci U S A *95*, 8858-8863.

Pierce, A. M., Schneider-Broussard, R., Gimenez-Conti, I. B., Russell, J. L., Conti, C. J., and Johnson, D. G. (1999). E2F1 has both oncogenic and tumor-suppressive properties in a transgenic model. Mol Cell Biol *19*, 6408-6414.

Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., *et al*. (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell *92*, 713-723.

Powers, J. T., Hong, S., Mayhew, C. N., Rogers, P. M., Knudsen, E. S., and Johnson, D. G. (2004). E2F1 uses the ATM signaling pathway to induce p53 and Chk2 phosphorylation and apoptosis. Mol Cancer Res *2*, 203-214.

Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A. (1986). Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature *322*, 78-80.

Ramirez, A., Bravo, A., Jorcano, J. L., and Vidal, M. (1994). Sequences 5' of the bovine keratin 5 gene direct tissue- and cell-type-specific expression of a lacZ gene in the adult and during development. Differentiation *58*, 53-64.

Reaper, P. M., di Fagagna, F., and Jackson, S. P. (2004). Activation of the DNA damage response by telomere attrition: a passage to cellular senescence. Cell Cycle *3*, 543-546.

Reed, S. I. (1992). The role of p34 kinases in the G1 to S-phase transition. Annu Rev Cell Biol *8*, 529-561.

Robertson, K. D., and Jones, P. A. (1998). The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. Mol Cell Biol *18*, 6457-6473.

Rodriguez-Puebla, M. L., LaCava, M., Gimenez-Conti, I. B., Johnson, D. G., and Conti, C. J. (1998). Deregulated expression of cell-cycle proteins during premalignant progression in SENCAR mouse skin. Oncogene *17*, 2251-2258.

Rogoff, H. A., Pickering, M. T., Debatis, M. E., Jones, S., and Kowalik, T. F. (2002). E2F1 induces phosphorylation of p53 that is coincident with p53 accumulation and apoptosis. Mol Cell Biol *22*, 5308-5318.

Rogoff, H. A., Pickering, M. T., Frame, F. M., Debatis, M. E., Sanchez, Y., Jones, S., and Kowalik, T. F. (2004). Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2. Mol Cell Biol *24*, 2968-2977.

Russell, J. L., Powers, J. T., Rounbehler, R. J., Rogers, P. M., Conti, C. J., and Johnson, D. G. (2002). ARF differentially modulates apoptosis induced by E2F1 and Myc. Mol Cell Biol *22*, 1360-1368.

Saavedra, H. I., Wu, L., de Bruin, A., Timmers, C., Rosol, T. J., Weinstein, M., Robinson, M. L., and Leone, G. (2002). Specificity of E2F1, E2F2, and E2F3 in mediating phenotypes induced by loss of Rb. Cell Growth Differ *13*, 215-225.

Schwarz, J. K., Bassing, C. H., Kovesdi, I., Datto, M. B., Blazing, M., George, S., Wang, X. F., and Nevins, J. R. (1995). Expression of the E2F1 transcription factor overcomes type beta transforming growth factor-mediated growth suppression. Proc Natl Acad Sci U S A *92*, 483-487.

Sears, R., Ohtani, K., and Nevins, J. R. (1997). Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. Mol Cell Biol *17*, 5227-5235.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Shan, B., and Lee, W. H. (1994). Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol Cell Biol *14*, 8166-8173.

Sherr, C. J. (1996). Cancer cell cycles. Science *274*, 1672-1677.

Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*, 1501-1512.

Shiloh, Y., and Kastan, M. B. (2001). ATM: genome stability, neuronal development, and cancer cross paths. Adv Cancer Res *83*, 209-254.

Singh, P., Wong, S. H., and Hong, W. (1994). Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. Embo J *13*, 3329-3338.

Stiewe, T., and Putzer, B. M. (2000). Role of the p53-homologue p73 in E2F1-induced apoptosis. Nat Genet *26*, 464-469.

Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. Embo J *17*, 5001-5014.

Stucki, M., and Jackson, S. P. (2004). MDC1/NFBD1: a key regulator of the DNA damage response in higher eukaryotes. DNA Repair (Amst) *3*, 953-957.

Sugimoto, M., Kuo, M. L., Roussel, M. F., and Sherr, C. J. (2003). Nucleolar Arf tumor suppressor inhibits ribosomal RNA processing. Mol Cell *11*, 415-424.

Suzuki, H., Kurita, M., Mizumoto, K., Nishimoto, I., Ogata, E., and Matsuoka, M. (2003). p19ARF-induced p53-independent apoptosis largely occurs through BAX. Biochem Biophys Res Commun *312*, 1273-1277.

Tao, W., and Levine, A. J. (1999). P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. Proc Natl Acad Sci U S A *96*, 6937-6941.

Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev *13*, 152-157.

Tolbert, D., Lu, X., Yin, C., Tantama, M., and Van Dyke, T. (2002). p19(ARF) is dispensable for oncogenic stress-induced p53-mediated apoptosis and tumor suppression in vivo. Mol Cell Biol *22*, 370-377.

Tsai, K. Y., Hu, Y., Macleod, K. F., Crowley, D., Yamasaki, L., and Jacks, T. (1998). Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. Mol Cell *2*, 293-304.

Tsai, K. Y., MacPherson, D., Rubinson, D. A., Crowley, D., and Jacks, T. (2002). ARF is not required for apoptosis in Rb mutant mouse embryos. Curr Biol *12*, 159-163.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T. K., Hampton, G. M., and Wahl, G. M. (2002). c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell *9*, 1031-1044.

Vigo, E., Muller, H., Prosperini, E., Hateboer, G., Cartwright, P., Moroni, M. C., and Helin, K. (1999). CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. Mol Cell Biol *19*, 6379-6395.

Wang, A., Pierce, A., Judson-Kremer, K., Gaddis, S., Aldaz, C. M., Johnson, D. G., and MacLeod, M. C. (1999). Rapid analysis of gene expression (RAGE) facilitates universal expression profiling. Nucleic Acids Res *27*, 4609-4618.

Wang, D., Russell, J. L., and Johnson, D. G. (2000). E2F4 and E2F1 have similar proliferative properties but different apoptotic and oncogenic properties in vivo. Mol Cell Biol *20*, 3417-3424.

Wang, X., Arooz, T., Siu, W. Y., Chiu, C. H., Lau, A., Yamashita, K., and Poon, R. Y. (2001). MDM2 and MDMX can interact differently with ARF and members of the p53 family. FEBS Lett *490*, 202-208.

Weber, J. D., Jeffers, J. R., Rehg, J. E., Randle, D. H., Lozano, G., Roussel, M. F., Sherr, C. J., and Zambetti, G. P. (2000). p53-independent functions of the p19(ARF) tumor suppressor. Genes Dev *14*, 2358-2365.

Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. Nat Cell Biol *1*, 20-26.

Weinberg, R. A. (1992). The retinoblastoma gene and gene product. Cancer Surv *12*, 43-57.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Wells, J., Boyd, K. E., Fry, C. J., Bartley, S. M., and Farnham, P. J. (2000). Target gene specificity of E2F and pocket protein family members in living cells. Mol Cell Biol *20*, 5797-5807.

Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature *334*, 124-129.

Wu, C. L., Zukerberg, L. R., Ngwu, C., Harlow, E., and Lees, J. A. (1995). In vivo association of E2F and DP family proteins. Mol Cell Biol *15*, 2536-2546.

Wu, L., Timmers, C., Maiti, B., Saavedra, H. I., Sang, L., Chong, G. T., Nuckolls, F., Giangrande, P., Wright, F. A., Field, S. J., *et al.* (2001). The E2F1-3 transcription factors are essential for cellular proliferation. Nature *414*, 457-462.

Wu, X., and Levine, A. J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. Proc Natl Acad Sci U S A *91*, 3602-3606.

Xie, S., Wu, H., Wang, Q., Cogswell, J. P., Husain, I., Conn, C., Stambrook, P., Jhanwar-Uniyal, M., and Dai, W. (2001). Plk3 functionally links DNA damage to cell cycle arrest and apoptosis at least in part via the p53 pathway. J Biol Chem *276*, 43305-43312.

Xu, G., Livingston, D. M., and Krek, W. (1995). Multiple members of the E2F transcription factor family are the products of oncogenes. Proc Natl Acad Sci U S A *92*, 1357-1361.

Yuan, Z. M., Shioya, H., Ishiko, T., Sun, X., Gu, J., Huang, Y. Y., Lu, H., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1999). p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. Nature *399*, 814-817.

Yuspa, S. H. (1998). The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. J Dermatol Sci *17*, 1-7.

Zhang, S. Y., Liu, S. C., Goodrow, T., Morris, R., and Klein-Szanto, A. J. (1997). Increased expression of G1 cyclins and cyclin-dependent kinases during tumor progression of chemically induced mouse skin neoplasms. Mol Carcinog *18*, 142-152.

Zhang, Y., and Xiong, Y. (1999). Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. Mol Cell *3*, 579-591.

Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell *92*, 725-734.

Ziebold, U., Lee, E. Y., Bronson, R. T., and Lees, J. A. (2003). E2F3 loss has opposing effects on different pRB-deficient tumors, resulting in suppression of pituitary tumors but metastasis of medullary thyroid carcinomas. Mol Cell Biol *23*, 6542-6552.

Ziebold, U., Reza, T., Caron, A., and Lees, J. A. (2001). E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. Genes Dev *15*, 386-391.

Zinkel, S. S., Hurov, K. E., Ong, C., Abtahi, F. M., Gross, A., and Korsmeyer, S. J. (2005). A role for proapoptotic BID in the DNA-damage response. Cell *122*, 579-591.

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